Mycobacterium tuberculosis-specific T-cell responses in latent infection and active disease

DISSERTATION

zur Erlangung des akademischen Grades
Doctor rerum naturalium
(Dr. rer. nat.)

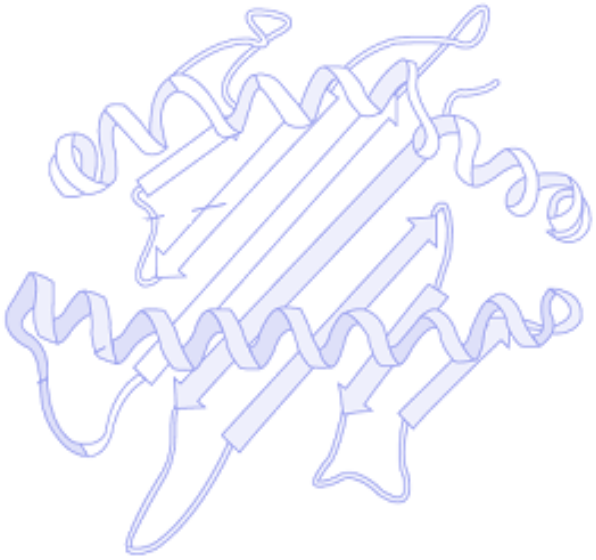
eingereicht an der
Mathematisch-Naturwissenschaftlichen Fakultät I
der Humboldt Universität zu Berlin

von
Diplom-Biologe
Sebastian D. Schuck
geboren am 22.05.1981 in Recklinghausen

Präsident der Humboldt-Universität zu Berlin
Prof. Dr. Christoph Markschies
Dekan der Mathematisch-Naturwissenschaftlichen Fakultät I
Prof. Dr. Lutz-Helmut Schön

Gutachter/-innen: 1. Prof. Dr. Hans-Dieter Volk
2. Prof. Dr. Stefan Kaufmann
3. Prof. Florian Kern

Tag der mündlichen Prüfung: 13. März 2009



Content

1 Abbreviations

This thesis uses the international one-letter amino acid code.

ADC	albumin, dextrose, catalase
Ag85a/b	Antigen85 a/b of *M. tuberculosis*
Alexa#	Alexa Fluor® #
AmCyan	Anemonia majano cyan fluorescent protein
APC	antigen presenting cell
APC	allophycoerythrin
APC-Cy7	allophycoerythrinCychrome7
ATP	adenosine tri-phosphate
BAK	BCG activated killer cells
BAL	bronchoalveolar lavage
BCG	*Mycobacterium bovis* bacillus Calmette Guérin
BCR	B-cell receptor
Bej	*M. tuberculosis* Beijing
BSA	bovine serum albumin
BSL3	biosafety level 3 (S3/L3 in Germany)
CD#	"cluster of differentiation" international nomenclature for cell surface molecules
CFU	colony forming units
CTL	cytotoxic T-lymphocyte
DC	dendritic cell
d	days
DNA	deoxyribonucleic acid
DosR	Dormancy survival regulator
dp	double positive
ER	endoplasmatic reticulum
E:T	effector:target ratio
eGFP	enhanced green-fluorescent-protein
ELISA	enzyme-linked immunosorbent assay

ESAT6_CFP10	6 kDa early secretory antigenic target_culture filtrate protein 10 fusion protein of *M. tuberculosis*
FACS	fluorescence activated cell sorting, flow cytometry
FCS	foetal calf serum
FITC	fluoreszeinisothiocyanat
FSC	forward scatter, in flow cytometry
GM-CSF	granulocyte-macrophage colony stimulating factor
h	hours
HIV	human immunodeficiency virus
Hsp#	heat shock protein # of *M. tuberculosis*
ICS	intracellular cytokine staining
IFNγ	interferon gamma
IL	interleukin
kDa	kilo-Dalton
LAK	lymphokine activated killer cell
LPS	lipopolysaccharide
LTBI	latent tuberculosis infection
M	mol/L
M. tuberculosis	*Mycobacterium tuberculosis*
mAb	monoclonal antibody
MF	macrophages
MHC	major histocompatibility complex
min	minutes
MOI	multiplicity of infection
MTB-lysate	M. tuberculosis whole cell lysate
NO	nitric oxide
NK	natural killer cell
OADC	oleic acid, albumin, dextrose, catalase
OD#	optical density, # indicates wavelength in nm
PAMP	pathogen associated molecular pattern
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline

PCR	polymerase chain reaction
Pe	phycoerythrin
Pe-Cy5	phycoerythrin-Cychrome5
Pe-Cy7	phycoerythrinCychrome7
PerCP	peridinin-chlorophyll-protein complex
PerCP-Cy5.5	peridinin-chlorophyll-protein complex-Cychrome5.5
Pen	Penicillin
PHA	phytohemaglutinin from *Phaseolus vulgaris*
PPD	purified protein derivative of *M. tuberculosis*
Rif	rifampicine
RNI	reactive nitrogen intermediates
ROI	reactive oxygen intermediates
Rpf	resuscitation promoting factor
rpm	rounds per minute
RT	room temperature
SEB	Staphylococcus enterotoxin B
SSC	sideward scatter
sp	single positive
Strep	Streptomycin
TAP	transporter associated with antigen presentation
TB	tuberculosis
tp	triple positive
TCR	T-cell receptor
T_h1	T-helper cell type 1
T_h17	T-helper cell type 17
T_h2	T-helper cell type 2
TNFα	tumour necrosis factor alpha
TLR	Toll like receptor
T_{reg}	T-regulatory cell
TST	tuberculin skin test
v/v	volume per volume
w/v	weight per volume

2 Abstract

Adaptive immune responses to *Mycobacterium tuberculosis* (*M. tuberculosis*) are crucial for an efficient containment of the pathogen and protection against secondary tuberculosis (TB). Although key mediators like the T_h1 cytokines IFNγ and TNFα released by *M. tuberculosis*-specific T cells are known, the immunological correlates determining the outcome of infection remain elusive. The vast majority of infected individuals is able to control TB by cellular immunity leading to latent tuberculosis infection (LTBI) while only ~10% will develop active disease. A better understanding of the underlying immune processes and the identification of protective biomarkers for TB are central aims of this thesis.

To address these topics adaptive immune responses to *M. tuberculosis* were analyzed in healthy LTBI and patients with active pulmonary TB. The recognition of *M. tuberculosis* derived antigens was studied by measuring the expression of IFNγ in $CD4^+$ $CD45RO^+$ memory T cells. A special hallmark was the inclusion of latency proteins associated with dormancy, reactivation and resuscitation of the pathogen. Optimized assay conditions were needed to detect T-cell responses towards latency-associated antigens by intracellular cytokine staining (ICS). This type of response cannot be detected by conventional short-term restimulation.

Seven days (d) *in vitro* incubation of PBMC and two rounds of restimulation followed by FACS analysis revealed T cell mediated recognition of the majority of tested latency-associated proteins in donors with LTBI. Comparison between active TB and LTBI documented significantly higher T-cell responses against 7 of 35 tested *M. tuberculosis* latency-associated antigens in LTBI.

Notably, T cells specific for one *M. tuberculosis* antigen, namely Rv3407, were exclusively detected in the subgroup of LTBI. Discrimination analysis revealed that the T-cell response against selected antigens with our novel assay is capable of distinguishing TB patients and LTBI with 82% accuracy using cross-validation. Again Rv3407 was by far the most influential component present in this cluster. Peptide pool stimulation in a similar fashion identified single distinct candidate epitopes within Rv3407 in four LTBI. Comparison of T-cell responses after short-term versus long-term stimulation demonstrated different prerequisites for *in vitro* restimulation between common immunodominant and latency-associated *M. tuberculosis* antigens. These differences argue for a different maturation stage /

cellular phenotype of $CD4^+$ $CD45RO^+$ memory T cells recognizing latency-associate *M. tuberculosis* antigens.

Additionally, cellular cytokine secretion patterns of *M. tuberculosis*-specific T cells were studied to evaluate the quality of adaptive immune responses contributing to protection against TB. This analysis documented an increased amount of poly-cytokine producing T cells in LTBI compared to active TB. Especially the amount of IFNγ/TNFα double positive (dp) and IFNγ/TNFα/IL2 triple positive (tp) T cells was affected and treatment with low doses of IL7 further increased this T-cell proportion. The *in vivo* relevance of poly-functional T cell subsets cannot be addressed by cross-sectional clinical studies and lead to the establishment of a novel *in vitro* killing assay based on flow cytometry.

The infection of human monocyte-derived macrophages with enhanced green fluorescent protein (eGFP) expressing mycobacteria allowed the investigation of functional properties of different autologous T-cell subsets. Preliminary experiments proofed the potential of the new assay to quantify intracellular mycobacteria by measuring the amount of $eGFP^+$ macrophages directly. Moreover, the *in vitro* assay revealed dose dependent killing of intracellular mycobacteria after co-culture with autologous PBMC activated by *M. tuberculosis* whole cell lysate and IL2. The determination of viable macrophages by flow cytometry after co-culture documented fundamental differences regarding the killing mechanism carried out by both types of effector PBMC. The collateral damage to macrophages varied significantly depending on the stimuli used for activation of autologous PBMC. Clearly such differences would have been missed by conventional plating of mycobacteria to determine bacterial titers. Nevertheless, and probably due to heterogeneity within the macrophage population, substantial assay to assay variations afflict the potential use of the novel assay to study host / pathogen interactions *in vitro*.

3 Introduction

3.1 The immune system

The mammalian immune system plays an important role in tumor surveillance, but its major aim is protection of the host against invading microorganisms and pathogens. Due to the broad variety of possible targets like viruses, bacteria, fungi, protozoa and parasites, mammals evolved complex defense mechanisms which comprise soluble (humoral) as well as cellular components. The immune system can be separated into the innate and the adaptive part. While the innate immune system is based on recognition of conserved features of the invading organism, the adaptive immune system is able to specifically target unique structures. Despite all mechanistic differences, both arms of the immune system are intertwined in order to provide efficient and long lasting protection.

3.1.1 Innate immune responses

The innate immune system reacts independently from immunological memory and is initiated immediately after contact with foreign antigens. The type as well as the kinetics of the response is not altered by re-encountering the same pathogen. The advantage of such a pre-programmed system is the ability to combat foreign organisms immediately without inducing more specific ways of host defense. The rapid reactivity of the innate immune system predestines it as a “first line of defense”, since full activation can be achieved without re-encountering the antigen. Consequently most of the invading organisms are cleared from the body without the help of adaptive immune responses (Medzhitov and Janeway, 2000). In order to fulfill these requirements the responsible receptors of the innate immune system sensing “non-self” antigens are germ line-encoded with a genetically predetermined specificity. These receptors (e.g. Toll-like receptors; TLRs) recognize conserved pathogen-associated molecular patterns (PAMPs) present amon groups of microorganisms.

Classically such molecular targets are lipopolysaccharides (LPS) of Gram-negative bacteria and lipoteichoic acids of Gram-positive bacteria (recognized by TLR-4 and TLR-2), respectively. Double stranded RNA from certain viruses (TLR-3) as well as flagellin of flagella expressing bacteria (TLR-5) also represent important target structures.

Microbial DNA is recognized by TLR-9 as unmethylated cytosine–guanosine dinucleotide sequences flanked 5' by two purines and 3' by two pyrimidines (CpG motifs). Hence pattern

recognition receptors are able to discriminate between different types of pathogens resulting in the activation of conserved host defense signaling pathways. For TB the involvement of TLR-2 as well as TLR-4 has been proposed (Means, et al., 1999). At least for TLR-2 which can recognize the *M. tuberculosis* derived 19kDa lipoprotein a role in innate host defence against TB could be demonstrated (Reiling, et al., 2002).

TLR mediated secretion of inflammatory cytokines plays an important role since it stimulates potent presentation of antigens which cross-links the innate immune system to adaptive immune responses. Recognition of microorganisms leads to a diversity of innate cellular defense mechanisms like phagocytosis, secretion of signaling molecules or release of toxic granules. Efficient effector cells of the innate immune system are neutrophils, dendritic cells (DC) and macrophages. Neutrophils patrol the body in high numbers and in most cases they are the first immune cells encountering an invading pathogen, followed by monocytes which migrate more slowly to the side of infection. Consequently these immune cells are have the first contact to M. tuberculosis after airborne infection.

Both cell types are able to engulf and kill invading pathogens by phagocytosis. For that purpose macrophages possess specialized receptors like the mannose receptor recognizing carbohydrates which are normally not exposed on the surface of mammalian cells in order to discriminate between “self” and “non-self” molecules (Stahl and Ezekowitz, 1998). Another example is the so called scavenger receptor binding to modified low density lipoproteins (Bowdish, et al., 2007). Additional phagocytic receptors expressed on macrophages and neutrophils are specific for antibodies and complement allowing enhanced phagocytosis of opsonized pathogens. The uptake of mycobacteria is very diverse and involves a variety of receptors which have different consequences on the immune response (see *3.2.2 The immune response against M. tuberculosis*).

DC also take up pathogens or cell debris but their main function is to process foreign antigens and present them to cells of the adaptive immune system. Finally Natural-Killer (NK) cells are important for the removal of virus infected or malignantly transformed host cells by recognizing the “altered self” stage of their target cells (Guevara-Patino, et al., 2003).

Since inflammatory responses and the underlying reaction cascades also damage host tissue and cause necrosis, the activation of the innate immune system has to be tightly controlled. Macrophages for instance require danger signals, e.g. activation through TLR or cytokine receptors to develop their full antimicrobial potential. Crucial in this process is interferon γ

(IFNγ) since it potently activates macrophages and the resulting production of toxic molecules enhances killing of engulfed microorganisms. The group of effector molecules includes reactive oxygen intermediates (ROI), reactive nitric oxide intermediates (RNI), antimicrobial peptides and lysozyme. Simultaneously activated macrophages secrete different cytokines like tumor necrosis factor α (TNFα), interleukin 12 (IL-12), interleukin 18 (IL-18) and various chemokines. All together synergistically lead to local inflammation and migration of innate as well as adaptive immune cells to the side of infection. Here TNFα is a very efficient inducer of other chemokines and their receptors. Up-regulation of E-selectin, intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) by endothelial cells allow leukocytes to leave the blood vessel and migrate into the tissue (extravasation). By chemotaxis the leukocytes are able to travel along the chemokine gradient and finally reach the inflammatory side (Janeway, 2005). Especially for the host response against *M. tuberculosis*, IFNγ together with TNFα can be considered to be key cytokines since they allow killing of intracellular mycobacteria and potently promote the activation of infected phagocytes.

In addition to the induction of inflammation, cytokines are also needed for the generation of adaptive immune responses. As mentioned above the DC is an important connection between the innate and the adaptive immune system. Immature DC constantly endocytose soluble and particular antigens as well as apoptotic material (Schaible, et al., 2003). However inflammatory signals (IFNγ and/or TLR activation) are required for maturation of the DC to become an antigen presenting cell (APC). In the course of differentiation it exposes foreign peptides loaded onto MHC-molecules (major histocompatibility complex) on its surface and migrates to the draining lymph node. In addition, the mature DC increases the surface expression of co-stimulatory molecules like CD40, CD80 and CD86. They provide appropriate signals for activation of antigen-specific T cells of the adaptive immune system. This takes place in the specialized environment of the secondary lymphoid tissue where naïve T cells “scan” MHC-peptide complexes on mature DC with their unique T-cell receptors (TCR). The T cell with the appropriate TCR for the foreign peptide-MHC complex will proliferate and mount a specific adaptive immune response.

3.1.2 Adaptive immune responses

In contrast to the predetermined receptors of the innate immune system, the specificity and the "target structures" of the adaptive immune system are not germ line encoded. They evolve constantly by genetic rearrangement, clonal selection and hypermutation (only B cells) (Janeway, 2005). Despite the limited repertoire of genes, the diversity of adaptive receptors (BCR and TCR) even exceeds the whole number of host genes. This is achieved by recombination of different gene segments on a cellular level during development, covering the plethora of possible epitopes (Delves and Roitt, 2000). The broad epitope repertoire of the adaptive immune system comprehends the risk of generating self-reactive lymphocytes leading to autoimmunity. Therefore auto-reactive lymphocytes are negatively selected during development in primary lymphoid organs. In addition, T cells and B cells recognizing their cognate antigen without co-stimulatory "danger signals" become apoptotic or anergic and lose their effector functions (Wucherpfennig and Strominger, 1995). Another mechanism to avoid autoimmunity and to shut down inflammatory processes and is carried out by specialized regulatory T cells (T_{reg}) which will be described in further detail later on.

The major effectors of the acquired immune system are B- and T cells. While B cells recognize soluble antigens directly via the B-cell receptor (BCR), a membrane bound variant of an antibody, T cells depend on epitopes presented within MHC-peptide-complexes. This means that B cells can sense discontinuous (steric) epitopes while T cells are limited to linear epitopes. Both cell types undergo activation and clonal expansion after encountering their cognate antigen during infection. After antigen-specific proliferation the B cell transforms into an immunoglobulin M (IgM) secreting plasma cell. Somatic hypermutation further increases the affinity of the antibodies and B cells are able to produce antibodies of different isotypes (class switch) by T-cell help. Only a small fraction of the expanded B cells will survive the so-called contraction phase after fighting down the infection and contributes to a long lasting memory. This allows the adaptive immune system to react rapidly after re-encountering the cognate antigen since memory cells do not follow the kinetic of naïve lymphocytes and can proliferate directly leading to the instant generation of effector cells.
T-cells follow a comparable kinetic but possess different features and requirements. Their activation depends on proper presentation of antigens by MHC molecules and can be influenced by the cytokine signature accompanying infection (cytokine imprint). MHC class I

molecules present their cargo (in most cases peptides) to $CD8^+$ T cells while loaded MHC class II molecules interact with T cells carrying the CD4 co-receptor. The MHC molecules are not equally distributed since class I is present on every host cell while class II is restricted to professional APC.

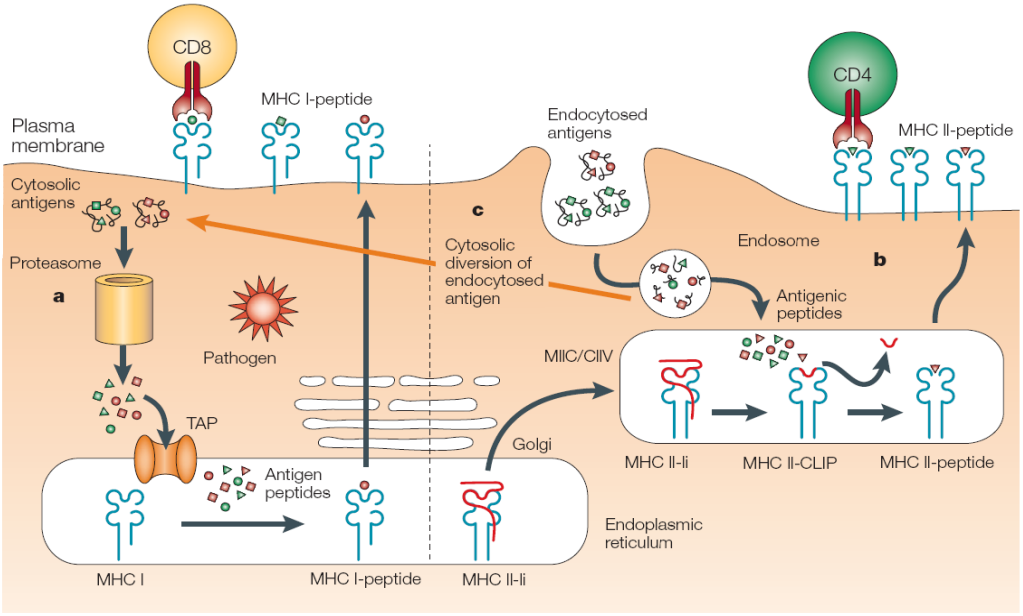


Fig. 1: Antigen presentation pathways.

Molecular mechanism for presentation of endogenous and exogenous antigens. **a)** Endogenous proteins are degraded by the proteasome and short peptides are actively transported into the lumen of the ER by TAP molecules. Peptides are loaded onto MHC-I molecules, reach the cell surface via the secretory pathway and can be presented to $CD8^+$ T cells. **b)** Exogenous antigens are taken up by endocytosis, degraded inside the endosomes and finally antigenic peptides reach the MIIC/CIIV compartment. Here appropriate peptides can be loaded onto activated MHC-II complexes and are presented to $CD4^+$ T cells on the cell surface. **c)** By cross presentation endogenous antigens can reach the MHC-I pathway and will be presented to $CD8^+$ T cells. According to (Heath and Carbone, 2001)

Classically cytosolic antigens, e.g. viral proteins, are degraded by the proteasome and small peptides are then shuttled into the lumen of the endoplasmatic reticulum (ER) by transporters associated with antigen processing (TAP). TAP is a member of the ABC transporter family and is a heterodimeric multimembrane-spanning polypeptide consisting of TAP1 and TAP2. The two subunits form a peptide binding site and two ATP binding sites that face the lumen of the cytosol. Inside the ER peptides which fulfill special criteria (anchor amino acids at position 2 and 9) are loaded onto MHC-I complexes. MHC class I molecules are heterodimers, consisting of a single transmembrane polypeptide chain (the α-chain) and a soluble β_2 microglobulin (which is not encoded within the MHC gene locus). The α chain has three polymorphic domains, α_1, α_2, α_3. Between α_1 and α_2 is the peptide-binding groove which

binds peptides derived from cytosolic proteins with a typical length of 8-9 amino acids. After assembly of the MHC-peptide complex the molecule reaches the cell surface via the secretory pathway. This passage includes a number of post-translational modifications, especially regarding N-glycans which mature inside the Golgi apparatus.
In contrast to MHC-I molecules, the invariant chain (Ii) prevents MHC class II molecules from being loaded inside the lumen of the ER. Like MHC class I molecules, class II molecules are heterodimers, but they consist of two homologous peptides, an α and β chain, both of which are encoded in the MHC. Because the antigen-binding groove of MHC class II molecules is open at both ends it binds longer peptides with a typical size of 15-24 amino acids. The corresponding groove on class I molecules is completely embedded leading to the binding of shorter peptides. After passage of MHC-II-Ii complexes through the Golgi apparatus they reach the MIIC/CIIV compartment and the invariant chain is degraded. MHC-II complexes are now able to bind antigenic peptides which enter the APC via the endocytic route. However the dogma of endogenous antigens presented via MHC-I and exogenous antigens presented via MHC-II does not hold true, because DC for instance can transfer exogenous antigens from other cells to their own MHC-I pathway in a process called cross presentation (Rock and Shen, 2005; Winau, et al., 2004). Although the details of this pathway remain elusive, specialized subcellular compartments bearing characteristics of both, the endoplasmic reticulum and the endosome, seem to be involved (Guermonprez, et al., 2003). TAP dependent TAP independent processes are crucial for this process which is in the focus of present immunological research (Burgdorf, et al., 2008). Since *M. tuberculosis* is internalized by phagocytes and resides inside a membrane bound vesicle, the most prominent T-cell response is carried out by $CD4^+$ T cells which recognize mycobacterial antigens via MHC-II. Interestingly cross presentation to $CD8^+$ T cells seem to be play a role in long term immunity to TB. It could be shown that apoptotic vesicles harbouring mycobacterial antigens are taken up by APC and lead to priming of $CD8^+$ T cells (Schaible, et al., 2003).

Once activated and expanded the effector cells of the adaptive immune system possess a functional diversity to fulfil their specific role. According to the recognition of cytosolic antigens the major task of $CD8^+$ T cells is T-cell mediated killing of infected host cells. Such cytotoxic T lymphocytes (CTL) are important for the removal of virus infected cells, but also contribute to the defence against other intracellular pathogens as well as malignantly

transformed host cells (Bevan, 2004). CTL either induce programmed cell death by FAS (factor activating ExoS) – FAS ligand interaction (apoptosis) or initiate direct killing by the release of cytotoxic molecules like granzyme, perforin or granolysin (necrosis) (Barry and Bleackley, 2002; Masson, et al., 1986; Stalder, et al., 1994). These cytotoxic effector molecules can also act on intracellular pathogens. After killing of their host cell they are exposed to other components of the humoral and cellular immunity.

The destiny of a $CD4^+$ T cell after antigen-specific activation is even more diverse. The differentiation is influenced by the co-stimulatory signals as well as the cytokine environment produced by cells of the innate immune system upon infection (Feili-Hariri, et al., 2005). Today four distinct $CD4^+$ T-cell lineages with different functional features are known – namely the T_h1, T_h2, T_h17 and the T_{reg} lineage.

T-helper cells type 1 (T_h1) are normally induced by IL12 or IFNγ during the early phase of an infection and act as pro-inflammatory mediators of the immune system. They are involved in the defence against many intracellular bacteria and protozoa since T_h1 cells are potent activators of phagocytes, especially macrophages. Hereby the major effector cytokines are IFNγ and TNFα which synergistically stimulate macrophages and influence adjacent T cells to differentiate into T_h1 cells (polarization). IL2 is also produced by activated T_h1 cells. It serves as a growth factor for T cells in the periphery and supports the expression of other effector cytokines. Another important cytokine in this context is the granulocyte-macrophage colony stimulating factor (GM-CSF) which enhances microbicidal activity, oxidative metabolism and phagocytotic activity of neutrophils and macrophages. It also improves the cytotoxicity of these cells. More cytokines and chemokines are known to be produced by T_h1 cells representing the complexity of adaptive immune responses. All together the set of T_h1 related cytokines promote inflammation and cell mediated cytotoxic responses.

The counterpart of the T_h1 is the T_h2 lineage which promotes a different cytokine milieu leading to humoral instead of cellular immune responses. T_h2 cells are generated after encountering IL4 during interaction with the APC and react with the secretion of various anti-inflammatory cytokines beneficial for humoral immune responses (antibody production). By release of IL4, IL5 and IL13 T helper cells of the T_h2 subset activate mast cells, basophils as well as eosinophils which play a role in the defense against helminths (Cherwinski, et al., 1987; Janeway, 2005; Jankovic, et al., 2001; Mosmann, et al., 1986; Mosmann and Coffman, 1989; Romagnani, 1991). Although T_h1 ad T_h2 lineages represent antagonistic arms of the

adaptive immune system, many examples are known where both lineages are influenced and regulated (Maziak, 2003; Nabel, 2002; Szabo, et al., 2003).

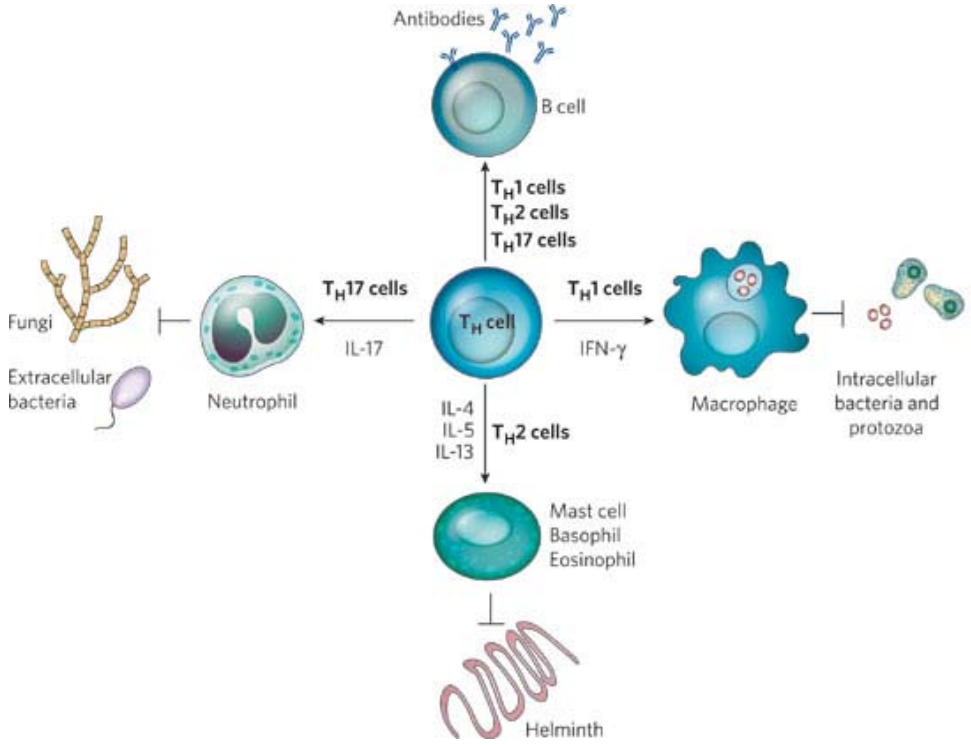


Fig. 2: T-cell lineages and their specific functions.

T_h1 and T_h2 cells are counter-balanced parts of the immune system leading to inflammation and cellular responses on the one hand and humoral immunity on the other hand. While T_h1 cells are able to activate macrophages (fighting intracellular bacteria / protozoa) T_h2 cells are involved in the defence against helminths. The newly discovered T_h17 cells represent another distinct lineage which activates neutrophils by the release of IL17 and IL22. Hereby T_h17 cells contribute to protection against extracellular pathogens. To a variable degree the help of all sub-lineages is needed to promote humoral immune responses by B cells. Suppressive Treg cells needed for a tight control of cellular immune responses are not shown in this overview. According to (Medzhitov, 2007).

T_{reg} cells mostly share the $CD4^+$ $CD25^+$ phenotype, but can also be $CD25^-$ or $CD8^+$. A more general feature is the expression of the transcriptional regulator forkhead box P3 (FoxP3). Their task is not only to maintain self-tolerance and homeostasis, T_{reg} cells are also needed to terminate the immune response properly after the successful clearance of the pathogen. Similar to conventional T cells T_{reg} cells develop in the thymus and expand in an antigen-specific manner. They have been shown to prevent expansion of classical effector T cells *in vivo* and inhibit T-cell activation *in vitro*. The suppressor mechanism of regulatory T cells is not clearly understood but interleukin 10 (IL10), transforming growth factor β (TGFβ) and cytotoxic T-lymphocyte associated molecule 4 (CTLA-4) seem to be involved (Annacker, et al., 2001; Fontenot, et al., 2003; Hori, et al., 2003; Read, et al., 2000; Shevach, 2000).

Finally an additional T helper cell subset has been identified: T_h17 cells were named according to the characteristic expression of IL17 upon specific stimulation. This cytokine fulfills an important effector function in host response to bacteria (e.g. the attraction of neutrophils to the site of infection) but can be also linked to autoimmunity (Kolls and Linden, 2004). In the mouse system, IL6 and TGFβ syngergize to induce T_h17 cells while their effector function is dependent on the presence of IL23 (Aggarwal, et al., 2003; Veldhoen, et al., 2006). In contrast to the murine system, the induction of human T_h17 cells depend on IL1β and IL6, but not on TGFβ (Acosta-Rodriguez, et al., 2007). Of several known innate immune stimuli, bacterial peptidoglycan (PGN) was most potent at eliciting IL-17 production. Probably muramyl dipeptide, a product of PGN catabolism and a ligand for NOD2 (nucleotide-binding oligomerization domain containing 2), is responsible for the observed production of effector cytokines. Remarkably NOD2 is often mutated in Crohn´s disease (CD), an autoimmune condition characterized by excessive intestinal inflammation (Meylan, et al., 2006). Moreover misregulation of IL-23 and the T_h17 axis have been implicated in the pathogenesis of CD (Duerr, et al., 2006). The role of different T-cell subsets as well as B cells in host defense against TB will be described in the next chapter. In brief, $CD4^+$ T cells of the T_h1 lineage are key players and absolutely required for control of bacterial growth while T_h2 cells have adverse effects on the outcome of infection (Caruso, et al., 1999; North, 1998). The function of T_h17 cells and B cells is not clearly understood. The absence of B cells within knock-out experiments in mice did not cause altered susceptibility to TB (Turner, et al., 2001). Although IL17 is an important factor in inflammation and attracts neutophils as well as macrophages, its absence did not lead to a server immunopahology as long as T_h1 cells are present (Khader and Cooper, 2008). Anyway T_h17 cells may be important to orchestrate the immune response to TB since vaccinated animals lacking this T-cell subset showed a loss of protection (Khader, et al., 2007).

3.2 Tuberculosis

M. tuberculosis, the etiologic agent of TB in humans, causes 9.2 million new infections every year and 1.7 million deaths annually (WHO, 2008). Especially in high incident countries like Africa or Asia TB, also known as the wasting disease, is a major reason for morbidity and death. Acute infection goes along with fever, night sweat, weight loss, fatigue, lassitude, rheumatic pains, coughing, gastroenteritis and lethargy. Due to estimations of the World

Health Organization up to one third of the world's population is latently infected with *M. tuberculosis*, underlining the fact that patients with acute disease only represent the tip of the iceberg (WHO, 2008).

3.2.1 History and modern weapons

Already in 1882 Robert Koch discovered the etiologic agent of TB which has been named *M. tuberculosis* later. The pathogen is a facultative intracellular bacterium with an extremely long doubling time up to 48 hours and a thick, waxy-like cell wall (Koch, 1882; Lehmann and Neumann, 1896). This unique cell wall contains a mycolic acid layer, connected via arabinogalactan polysaccharide to the conventional peptidoglycan layer (Barry, et al., 2007) and contributes to the special features of the pathogen to withstand host immune defense (see *3.2.2 The immune response to M. tuberculosis*).

The early approaches by Robert Koch to establish a vaccine against TB based on tuberculin were not successful (Brock, 1988). Soon thereafter two French researchers, Albert Calmette and Camille Guérin, attenuated the related cattle pathogen *Mycobacterium bovis* and delivered it as a live vaccine. In 1921 the so-called *M. bovis* bacillus Calmette Guérin (BCG) was introduced to the public and provided protection against childhood TB which is associated with severe courses of disease like TB meningitis (infection of the meninges by *M. tuberculosis*) or military TB (disseminated infection) (Clarke and Rudd, 1992). Despite all advantages BCG immunization was not very popular in the beginning and this became worse after the disaster in Lübeck where in 1927 more than 70 infants died due to a contamination of *M. tuberculosis* within the BCG preparation (Moses, 1930). Meanwhile BCG has become a successful live-vaccine against TB in newborn and infants, but it remain ineffective against adult pulmonary TB (Reece and Kaufmann, 2008).

Regarding the treatment of active disease a major breakthrough was the discovery of antibiotics and the usage of streptomycin to cure TB (Schatz, et al., 2005). Today the recommended drug regiment comprises four different first-line drugs which have to be taken for six up to nine months. These drugs are aiming at different molecular pathways of the pathogen: Isoniazid (Inh) exhibits its bactericidal effects by inhibition of cell wall mycolic acid synthesis and other basic metabolic activities. Rifampicine (Rif) inhibits specifically the RNA synthesis by targeting the RNA polymerase β subunit. Pyrazinamide (Pza) is a structural analogon of

nicotinamid and interferes with the membrane energy metabolism also leading to a disrupted membrane transport. The last drug in this scheme is ethambutol (Eth) which acts bacteriostatically by impairing the cell wall arabinogalactan synthesis (probably by blocking the underlying arabinosyl transferase which prevents cell wall arabinan polymerization) (Zhang and Amzel, 2002). In that regiment rifampicine is the only broad-spectrum drug since the other substances are TB specific. However the appearance of resistances against these first-line drugs either by incomplete treatment or spontaneous mutations is a serious problem. Multi drug resistant TB (MDR-TB) is characterized by lost effectiveness of at least isoniazid and rifampicine, the two most powerful anti-TB drugs (Zhang, 2005). The treatment requires prolonged and more expensive chemotherapy including second-line drugs, i.e. para-amino-salicylate, kanamycin, cycloserine, ethionamide, amikacin, capreomycin, thiacetazone and fluoroquinolones (Zhang and Amzel, 2002). Recently extensively drug resistant (XDR) strains were identified. This means that such bacilli are even resistant to at least three of the six classes of second-line drugs. A well known example is the study of Tugela Ferry (South Africa) monitoring 544 patients of whom 221 suffered from MDR-TB including 53 cases of XDR-TB (Singh, et al., 2007). This forced the WHO to strengthen its efforts on TB leading to the release of new guidelines for treatment (DOTS-plus), public campaigns as well as improvement of health care systems. An additional part of the global STOP TB strategy launched in 2006 was to develop better drugs, diagnostics and vaccines (Torrelles, et al., 2008; WHO, 2008).

3.2.2 The immune response against *M. tuberculosis*

The high rate of healthy individuals with LTBI can be explained by the typical course of infection. *M. tuberculosis* is being transmitted via aerosol from a person with active TB. By inhaling droplets the pathogen reaches the lung where it encounters the pulmonary innate immune system, the first line of defense against microbes entering via the respiratory route. As already mentioned the innate immunity is based on the broad recognition PAMPs. Due to the unique waxy cell wall structure of *M. tuberculosis* the pathogen is more resistant to soluble direct antimicrobial components like the pulmonary surfactant proteins A and D (Torrelles, et al., 2008). Consequently, phagocytes within the lung alveoli play a key role since their surface is rich in complement receptors and C-type lectin pattern recognition receptors (Torrelles, et al., 2008). Important for the internalization of *M. tuberculosis* are the

mannose-receptor, dendritic cell-specific ICAM-3-grabbing nonintegrin (DC-SIGN) and DC-associated C-type lectin-1 (Dectin-1) on bronchoalveolar macrophages. These receptors can directly or indirectly bind to sugar residues within the cell wall of the pathogen (Aderem and Underhill, 1999) and contribute to the propagation of pro-inflammatory signals (Jo, 2008). Interestingly the recognition of *M. tuberculosis* components via TLR triggers intracellular signaling cascades that promote inflammation, but can also trigger signals that dampen the innate immune response (Jo, 2008). Consequently the interaction of *M. tuberculosis* with pattern recognition receptors leads to activation or suppression of the host immune system, depending on the entry route and the accompanying signals.

The complement cascade is also involved in the uptake of mycobacteria. Activation via the alternative pathway leads to the deposition of C3b and C3bi on the bacterial surface which allows internalization by complement receptor 1 and 3 (Schlesinger, et al., 1990). Additionally the opsonization by C3b can be achieved by mechanisms which are specific for pathogenic mycobacteria, based on the formation of a C3 convertase after association of C2a with surface factors of the pathogen (Schorey, et al., 1997).

Once internalized, *M. tuberculosis* is contained inside a membrane bound vacuole. Classically the pathogen is believed to actively interfere with phagolysosomal fusion by immune evasion mechanisms (Hart, et al., 1972). It has been proposed that a lack of acidification based on the impaired recruitment of proton-ATPases is responsible for the inhibited maturation of phagosomes (Sturgill-Koszycki, et al., 1994). More recent studies could even show that *M. tuberculosis* is able to maintain intrabacterial pH under acidified lysosomal conditions (Vandal, et al., 2008). However, the pathogen is able to reside and proliferate inside these early phagosomes.

Within the alveolar phagocytes *M. tuberculosis* reaches the lung parenchyma and later the draining lymph nodes where macrophages and DC present mycobacterial antigens to T cells (Kaufmann and McMichael, 2005). At the site of infection dying macrophages attract monocytes and other immune cells leading to inflammation and formation of a local lesion. The whole process involves the release of TNFα and results in the secretion of chemokines as well as the up-regulation of chemokine receptors. The help of the adaptive immune system is needed to fully activate macrophages to kill intracellular mycobacteria (fig. 3). Important for the generation of an adaptive immune response are DC which can take up bacilli via DC-

SIGN. They are also crucial for cross-presentation of mycobacterial antigens to T and B cells by processing apoptotic vesicles (Winau, et al., 2006). Initial studies with B cell deficient mice could not demonstrate a higher susceptibility to aerogenic infection with *M. tuberculosis* than wildtype mice based on the survival time (Turner, et al., 2001). Nevertheless, more recent studies observed higher bacterial titers and an exacerbated immunopathology with enhanced recruitment of neutrophils in these knock-out mice compared to wildtype (Maglione, et al., 2007). Hence, the role of antibody-mediated immunity to TB in humans is not completely understood and needs further investigation.

In contrast $CD4^+$ as well as $CD8^+$ T cells are essential for the host defense against *M. tuberculosis* infection. Mouse strains deficient for HLA-II or CD4 molecules die shortly after *M. tuberculosis* infection (Caruso, et al., 1999) while *in vivo* depletion of $CD8^+$ T cells impairs the long-lasting containment of the pathogen rather than the acute immune response (van Pinxteren, et al., 2000). As already mentioned the cytokine milieu which is produced upon TLR activation and intracellular infection of macrophages/DC is dominated by TNFα and other pro-inflammatory cytokines like IL12 and IL18. This cytokine environment leads to the biased differentiation of $CD4^+$ T cells into T_h1 cells. Following recognition of cognate mycobacterial antigens via their TCR within the lymphoid tissue they undergo clonal expansion and maturation. Fully activated T_h1 T cells start to modify their homing receptors, migrate to the side of infection and directly exert effector functions after re-encountering their specific antigen presented on MHC-II complexes.

Major effector cytokines in this process released by T cells are IFNγ and TNFα. Both are actively secreted by effector $CD8^+$ T cells as well as $CD4^+$ T cells of the T_h1 lineage. IFNγ as well as TNFα have broad effects on mammalian host cells, but in case of *M. tuberculosis* infections their most important functions are activation of phagocytes and tissue remodeling.

The IFNγ receptor consists of two different membrane bound subunits (R1 and R2) which are ubiquitously expressed on mammalian cells with the exception of erythrocytes. After homo-dimerization of IFNγ-R1 the complex associates with IFNγ-R2 leading to Janus-Kinase (JAK) mediated activation of signal transducer and activator of transcription 1 (STAT1). Active STAT1 translocates to the nucleus and induces transcription of direct response genes and more than 40 transcription factors resulting in a cascade like regulation of secondary response genes (Foxwell, et al., 1992). Despite broad effects on cell cycle regulation and apoptosis IFNγ acts more specifically on phagocytes. The bactericidal capacity of macrophages is

increased by generation of RNI / ROS mediated by the induction of the inducible nitric oxide synthase (iNOS) and assembly of the enzyme complex phagocyte oxidase (phox), respectively. IFNγ signals also promote phagosome-lysosome fusion and hereby overcome the immune evasion mechanisms of *M. tuberculosis* (Boehm, et al., 1997; Bogdan, et al., 2000). A general feature of IFNγ-receptor signaling is the up-regulation of MHC-I molecules on immune cells as well as MHC-II on nucleated cells to improve antigen presentation. In parallel the processing of antigens is optimized by induction of specialized proteasome subunits (immunoproteasome) and increased levels of TAP molecules (Kloetzel, 2001). Additionally the expression of adhesion molecules like ICAM-1 on vascular endothelial cells is increased. Together with the INFγ induced release of IP10 (interferon inducible protein 10), I-TAC (interferon-inducible T-cell alpha chemoattractant / CXCL11) and Mig (monokine induced by gamma-interferon / CXCL9) this leads to infiltration of CXCR-3 expressing leukocytes (macrophages, neutrophils, activated T cells) into the tissue (Boztug, et al., 2002; Dufour, et al., 2002).

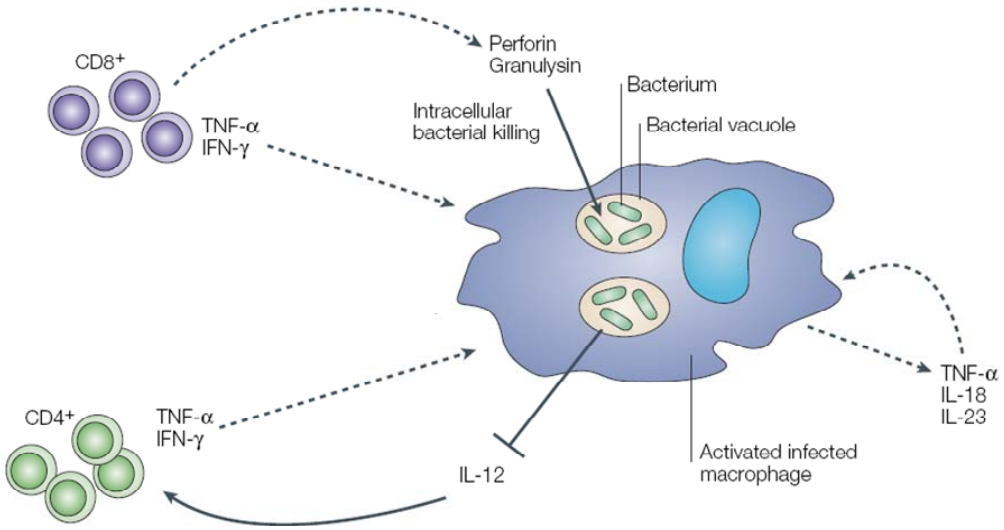


Fig. 3: Interaction of T cells with *M. tuberculosis* infected macrophages.

After phagocytosis *M. tuberculosis* resides in a unique membrane-bound vacuole and prevents phago-lysosomal fusion. To overcome this situation T-cell help and activation of the macrophage is required. Macrophage activation by IFNγ and TNFα promotes phagosomal maturation, vacuole acidification and the production of antimcrobial molecules, such as reactive nitrogen intermediates (RNI) by nitric oxide synthase 2(NOS2), reactive oxygen intermediates (ROI) or antimicrobial peptides. The release of the pro-inflamatory cytokines TNFα, IL18 and IL23 by activated macrophages also controls intracellular replication of mycobacteria, while IL12 promotes the secretion of IFNγ. Modified according to (Monack, et al., 2004).

The importance of IFNγ for host defense against infections becomes obvious in humans with hereditary IFNγ receptor deficiency. The ineffective immune response causes death by otherwise harmless intracellular bacteria. Even BCG vaccination in that scenario can be lethal (Jouanguy, et al., 1997). Various studies with knock-out mice also proved the fundamental role of IFNγ which in synergy with TNFα and IL18 orchestrates the immune system (Cooper, et al., 1993). TNFα is also released by T_h1 cells upon antigen-specific stimulation and can be secreted by macrophages/monocytes, neutrophils and NK cells after encountering bacterial lipopolysaccharides. The main receptors for TNFα are two high affinity subtypes (TNF-R1 and TNF-R2; also known as CD120a and CD120b) present on all somatic nucleated cells. The C-terminal intracellular region of TNF-R1 contains a so-called death domain, which is involved in signaling events leading to programmed cell death. The death domain of TNF-R1 interacts with a variety of other signaling adaptor molecules, including TRADD (TNF-R1-associated death domain protein) and RIP (receptor interacting protein) leading to the activation of the transcription factor NF-kappa-B (nuclear factor kappa B). NF-kappa-B responsive genes include those coding for a number of cytokines and growth factors, cytokine receptors, receptor signaling proteins, cell adhesion molecules and many other proteins involved in various processes (Baldwin, 1996). In resting macrophages TNFα induces the synthesis of IL1, prostaglandin E2 and stimulates phagocytosis. Like IFNγ TNFα causes up-regulation of cellular adhesion molecules and attracts immune cells to the side of infection. The dramatic influence of this key cytokine can be observed during anti-TNFα therapy in patients with arthritis leading to significant reactivation of TB in LTBI (Brassard, et al., 2006).

In summary, the local secretion of IFNγ and TNFα synergistically activates phagocytes to kill intracellular mycobacteria, attracts leukocytes (macropahges, neutrophils, activated T cells), promotes antigen processing and presentation as well as tissue remodeling.

3.2.3 Granuloma formation

Waves of infiltrating immune cells wall off infected macrophages and the resulting structure is called granuloma. It consists of central necrosis with is characterized by hypoxic conditions at later stages of infection. The granuloma contains *M. tuberculosis* infected phagocytes, surrounded by macrophages and leukocytes which probably try to kill the pathogen.

Consequently it has been speculated that inflammation, tissue damage and necrosis is limited to the granulomatous structure. The caseous granuloma can also be identified by X-ray examination and still contributes to diagnosis of clinical TB. The majority of infiltrating cells are $CD4^+$ T cells but also γδ T cells, unconventional CD1 restricted T cells and B cells are present within the granuloma and its periphery (Ulrichs and Kaufmann, 2006). The granuloma is a highly organized structure with a characteristic composition of cells present within different foci (fig. 4). The inner layer next to the central necrosis is rich in $CD4^+$ T cells as well as $CD68^+$ macrophages, while $CD8^+$ T cells are rare. Epithelioid cells and Langhans giant cells are also present in this area. The adjacent outer layer consists of massive lymphocyte infiltrations with high amounts of $CD4^+$ and $CD8^+$ T cells. While the inner layer seems to be the side of action for effector T cells and phagocytes, the outer layer is characterized by different lymphocyte subsets and may contribute to vigorous T cell priming.

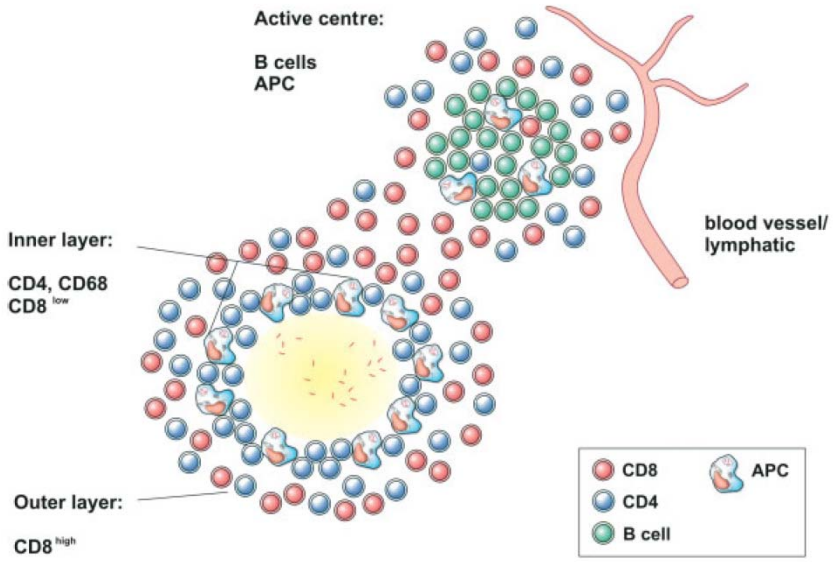


Fig. 4: Schematic overview of the lung granuloma architecture.

The hypoxic area of the central necrosis contains mycobacteria and oxidative stress signals drive *M. tuberculosis* into the dormant stage of its life cycle. The surrounding inner layer is rich in $CD4^+$ T cells as well as macrophages ($CD68^+$), while $CD8^+$ T cells are rare. The outer layer also harbors $CD8^+$ T cells and is less dense compared to the inner layer which walls of the central necrosis. Within the active centers in the periphery of the granuloma cross-talk of B-cells, T-cells and APC takes place. According to (Ulrichs, et al., 2004).

Interestingly the active centers surrounding the granuloma contain high amounts of B cells, T cells and *M. tuberculosis* infected APC. Histological analysis revealed that the B cells comprised different states of B-cell differentiation, containing both naïve ($CD23^+$) B cells and

memory ($CD27^+$) B cells as well as antibody-secreting plasma cells (Ulrichs, et al., 2004). The presence of different maturation stages is reminiscent of B-cell follicles in lymph nodes. Strong proliferation within the active centers and the outer layer of the granuloma in close proximity to professional APC emphasize the important role of granulomatous structures to promote cross-talk and priming of T and B cells. In the later stages of infection the granuloma will be surrounded by a fibrotic wall and gain a lymphoid follicular structure (Kahnert, et al., 2007). It has been proposed that the cell aggregates in the tissue surrounding granulomatous lesions in TB assume the structure and function of secondary lymphoid follicles, which ensure orchestration of the complex and long-lasting immune response to TB (Ulrichs, et al., 2004). Despite joint efforts of the innate and acquired immune system the host is not able to eradicate the pathogen completely. Oxidative stress signals in the central area drive the pathogen into the dormant stage of its life cycle and lead to the upregulation of dormancy-associated proteins. The shifdown of *M. tuberculosis* from active replication to dormancy can be separated into at least two steps. Within the first phase the pathogen changes from fast to slow replication while the second step is leading to a complete shutdown of replication (Wayne, 1994). Dormancy is characterized by low/absent metabolic activity and survival under hypoxic conditions. Dormant mycobacteria contribute to a long lasting reservoir of infectious bacteria leading to the latent stage of infection (*see 3.2.4 Latency versus active disease*).

3.2.4 Latency versus active disease

As already mentioned the vast majority of *M. tuberculosis* infected individuals will not develop active TB but remain latently infected for the rest of their lives. Only 5-10% of all infected individuals will suffer from active disease and 50% of them will develop TB within the first three years after infection. Although potent host defense mechanisms against *M. tuberculosis* and other intracellular pathogens exist, the course of diseases is marked by a long lasting balance between protective immune responses on the one hand and immune evasion of the bacilli on the other hand. Normally the primary infection takes place within the central part of the lung (Ghon complex) from where bacteria can spread through the lymphatic system to the blood and finally re-infect the lungs. This leads to the formation of secondary lesions and in case of immune-competent individuals the pathogen can be contained within granulomatous structures. Generally the primary infection can be fought without symptoms of

disease but for unknown reasons *M. tuberculosis* is not eradicated completely. The underlying mechanisms are still unclear, but *M. tuberclosis* possesses a variety of immune evasion strategies which seem to play a role.

Some mycobacterial genes like noxR1, noxR3, ahpC (Rv2428) and glbN (Rv1542c) are known to act against ROS and RNI either by neutralization or interference with metabolic pathways involved in generation of these toxic molecules (Bryk, et al., 2002; Ehrt, et al., 1997; Ouellet, et al., 2002; Ruan, et al., 1999). In this connection the phagosomal arrest is also important, although it can be reverted by IFNγ. Responsible is the block of acidification by exclusion of the vacuolar proton pump from the phagosomal membrane (Ferrari, et al., 1999) together with ESAT-6 (6kDa early secretory antigenic target) which interferes with the phagosomal membrane and causes membrane lysis (de Jonge, et al., 2007). Even antigen processing and presentation by APC can be influenced since various molecules of the pathogen lead to decreased surface expression of MHC-II molecules (Singh, et al., 2006).

A remarkable feature of *M. tuberculosis* is its ability to survive under hypoxic and nutrient-poor conditions (e.g. within the granuloma and activated macrophages) in a dormant stage which allows the pathogen to persist. This “life cycle” of the pathogen is characterized by extremely low metabolic activity and an altered proteomic profile. Important in this process are enzymes mediating an alternative energy metabolism [i.e. isocitrate lyase and glycine dehydrogenase; (Graham and Clark-Curtiss, 1999)] as well as thickening of the cell wall possibly mediated by PcaA, CmaA1, CmaA2 (Cole, et al., 1998) and/or the 16-kDa alpha crystalline (hspX) (Cunningham and Spreadbury, 1998)

To investigate the behavior of non-replicating mycobacteria under such conditions an *in vitro* model has been introduced by Wayne *et. al.* based on the incubation of *M. tuberculosis* in sealed containers leading to starvation and gradual depletion of oxygen (Wayne, 1994). Subsequent adaption of the bacilli leads first to a microaerophilic and later to an anaerobic stage with changes in gene expression as well as drug susceptibility (Wayne and Hayes, 1996).

A number of different factors is involved in the persistence under hypoxic conditions and transcriptomic microarray studies revealed a set of 48 genes being differentially regulated (Sherman, et al., 2001). Among this set of response genes a two-component transcription factor, namely Rv3133c, has been identified to be responsible for the induction of all these 48 genes and is therefore referred to as dormancy survival regulator (DosR) (Boon and Dick,

2002). By targeted deletion of the DosR locus it had been shown that DosR is essential for the pathogen response to hypoxia and needed for persistence (Park, et al., 2003). The genes regulated by DosR are clustered in nine distinct modules within the bacterial genome and form the so-called DosR regulon. Recently it has been shown that Rv3132c (DosS) and Rv2027c (DosT) encode sensor kinases which can phosphorylate DosR *in vitro*, thus initiating the dormancy response (Voskuil, et al., 2003). The sensor involved seem to be a heme-containing protein with specificity for oxygen as well as nitric oxide (Voskuil, et al., 2003).

Although the DosR regulon can be linked to virulence (present in *M. tuberculosis*, *M. smegmatis* and partially in BCG) there is no evidence so far about the metabolic stage of *M. tuberculosis* during latent infection. Latency simply describes an infected individual without symptoms of disease and does not refer to distinct stages of the pathogen life cycle. Since the tuberculous granuloma is characterized by hypoxia and bacterial numbers correlate well with the degree of oxygenation, it is still tempting to speculate that the majority of the bacilli are dormant and express DosR regulated genes.

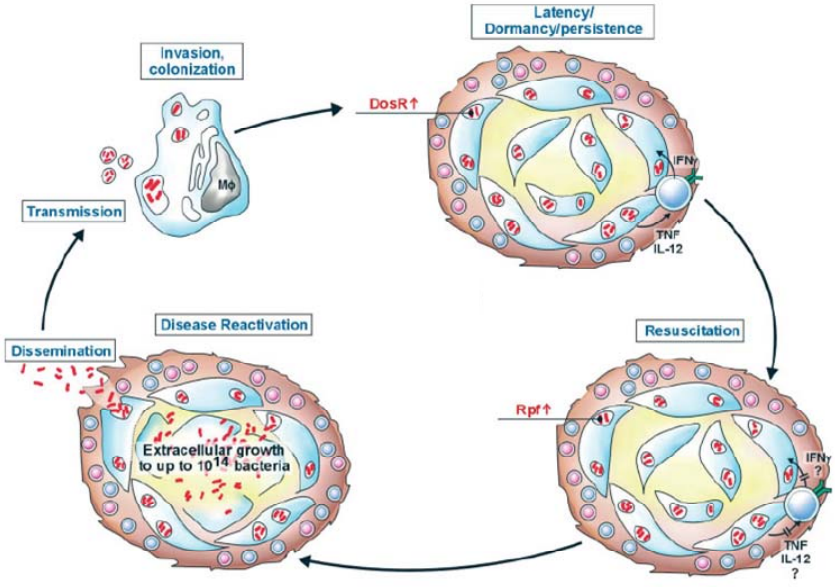


Fig. 5: The life cycle of *M. tuberculosis* and host/pathogen interactions.

The colonization of alveolar macrophages (MΦ) is the first stage upon airborne infection. Contribution of T-helper cells lead to the activation of phagocytes by IFNγ and TNFα which act synergistically. Next the formation of a tuberculous granuloma occurs and oxidative stress signals induce the expression of DosR encoded proteins. *M. tuberculosis* enters its dormancy stage characterized by slow or absent bacterial replication. After receiving appropriate "wake-up" signals the persisting bacteria up-regulate Rpf and become metabolic active again. Consequently replicating mycobacteria begin to disseminate leading to reactivation of disease. Modified according to (Ulrichs and Kaufmann, 2006).

In addition to mechanisms driving M. tuberculosis into dormancy it is fundamental to understand the processes which facilitate the recovery from dormancy and promote active replication. Various mechanisms have been discussed in the literature and it is known that culture supernatant can reverse dormancy (Sun and Zhang, 1999). In this connection a group of five secreted proteins has been identified recently (Biketov, et al., 2000).
One of these so-called resuscitation promoting factores (Rpf) shows homology to lysozymes and it has been speculated that peptidoylcan lysis and the resulting remodeling of the bacterial cell call could provide "wake up signals" (Cohen-Gonsaud, et al., 2005; Keep, et al., 2006).
Reactivation of TB in latently infected humans is not clearly understood, although various exogenous and endogenous factors promoting active disease are known (malnutrition, absence of medical care, drug abuse, age, HIV/AIDS, immunosuppressive therapy etc.). Most of them leading to an impaired adaptive immune response and consequently containment of the pathogen gets lost. But not in every case reactivation can be explained by a brake down of proper immune surveillance and it remains elusive whether appropriate "wake up signals" are needed or resuscitation is simply a matter of statistical probabilities (Chan and Flynn, 2004; Saunders and Britton, 2007).

3.2.5 Vaccines and biomarkers

The most efficient way to fight TB globally is the generation of improved vaccines which provide better protection against adult pulmonary TB than BCG does. As already mentioned BCG vaccination in newborns confers protection against severe forms of childhood TB (i.e. TB meningitis and miliary TB) but is less efficient in adults (Trunz, et al., 2006). As part of the global STOP TB initiative financial support has been provided for the development of new anti-TB vaccines. Basically five different strategies evolved: the use of recombinant BCG, generation of attenuated *M. tuberculosis* vaccine strains, subunit vaccines, viral vector delivery systems and DNA vaccination.
One recombinant BCG strain expressing the *M. tuberculosis*-specific antigen Ag85b is already in clinical trial phase I (Horwitz and Harth, 2003) and complementation of BCG with genes of the region of difference 1 (RD1) has also been reported (Pym, et al., 2003). Despite efforts to express otherwise absent or rare *M. tuberculosis* proteins it is also possible to alter the immune response elicited by BCG. This approach was followed by engineering a recombinant ureaseC deficient BCG strain expressing membrane-perforating listeriolysin. By

this means protein antigens should be able to pass from the phagolysosome to the cytosol leading to an increased generation of $CD8^+$ cytotoxic T-cell responses. Indeed induction of better protection against aerogenic challenge with *M. tuberculosis* than the parental strain could be shown in mice. Especially at late stages of infection this recombinant BCG was much more effective (Grode, et al., 2005).

Attenuated strains of *M. tuberculosis* have been developed and prominent examples are non-replicative auxotroph mutants with targeted deletions in genes involved in pantothenic acid synthesis (Sambandamurthy and Jacobs, 2005) and strains lacking the virulence associated gene PhoP (Asensio, et al., 2008). Due to the use of attenuated *M. tuberculosis* strains as live vaccines instead of complementing BCG with antigens safety issues may arise.

Subunit vaccines aim at boosting the BCG prime by delivering *M. tuberculosis* components (mostly proteins) together with an adjuvant. Latest subunit vaccines encompass Hybrid-1 (Ag85b_ESAT-6), HyVac-4 (Ag85b_TB10.4) and Mtb72F (Mtb39_Mtb32) which already passed clinical trial phase I (Dietrich, et al., 2005; Skeiky, et al., 2005; Weinrich Olsen, et al., 2001). Another possibility is the delivery of antigenic proteins by viral vectors; in this context a modified Vaccinia virus Ankara expressing Ag85a is already in phase I now (McShane, et al., 2004). Last but not least the principle of DNA vaccination can be combined with the other strategies to achieve better protection. In brief a DNA plasmid coding for antigenic proteins can be injected directly and is able to elicit robust T_h1, T_h2, CTL as well as antibody mediated immune responses (Tang, et al., 1992). The coding DNA fragment also contains a strong eukaryotic promoter, e.g. from cytomegalovirus, a terminator of transcription, e.g. the polyA sequence from bovine growth hormone, and components for expression and selection in prokaryotes during plasmid generation (i.e. origin of replication and resistances). Especially DNA vaccination against viral infections proved to be protective in animal models (Robinson, et al., 1993). Meanwhile a variety of DNA constructs have been tested alone or as part of a prime-boost scenario and most of these approaches confer more or less to protection in animal models (Orme, 2006).

Nevertheless and despite all efforts there is still no protective vaccine against adult pulmonary TB available. The beneficial effect of recent candidates which were selected due to reduced bacterial titers in animals has to be finally evaluated in humans. Another problem is the high number of LTBI since none of the new vaccines was efficient in post-exposure settings within animal experiments. It is worth to mention that all these candidates rely on classical

immunodominant antigens of *M. tuberculosis* which are expressed under normal, replicative conditions. The inclusion of antigens associated with dormancy, reactivation and resuscitation may be a promising vaccination strategy to drive the pathogen more into latency or even achieve complete bacterial clearance (Roupie, et al., 2007) (http://www.gcgh.org/CureInfection/Chal-lenges/ImmunologicalMethods/Pages/PostExposureTB.aspx).

Moreover the typical course of disease with a long lasting period of latency necessitates long term observations to follow the effect of vaccination. The direct analysis of vaccination success is still problematic since no molecular correlates of protection are known. This means that the induction of T-cell responses, the associated cytokine pattern as well as antibody levels can be measured easily but the contribution of such parameters to protection remains elusive. It has been proposed that the release of the key cytokine IFNγ by antigen-specific T cells could serve as a surrogate marker of protection, but more recent studies clearly showed that the magnitude of the adaptive immune response alone (measured by IFNγ) is insufficient (Mittrucker, et al., 2007). Therefore the hunt for new protective biomarkers for TB is still ongoing. Possible implications are not limited to vaccination studies; prognostic markers for the outcome of infection as well as therapeutic markers to monitor the success of treatment are urgently needed. Finally the diagnosis of clinical TB is based on X-ray examination, identification of the pathogen in patient's samples and modern interferon γ release assays (IGRA). Such IGRA detect antigen-specific memory responses of the host towards the unique protein antigens ESAT-6 and CFP10 of *M. tuberculosis*. Since these assays cannot discriminate between latent infection and active disease, a potent molecular biomarker would provide solutions for common day-to-day problems of physicians and patients in the field (Menzies, et al., 2007).

The situation is even more complicated since TB in animals is very different from human infection and unfortunately appropriate *in vivo* latency models are still missing. In mice for instance granuloma formation is different and the bacilli are able to replicate continuously without any dormancy. Even in more resistant mouse strains a persistent *M. tuberculosis* infection is characterized by high bacterial burden in various organs, which is in contrast to humans (Orme, 2003). A pragmatic solution could be the evaluation of beneficial and adverse effects of different T-cell signals and stimuli in *in vitro* assays. Such assays could directly measure the influence of T-cell help on the activation of infected phagocytes and their ability to kill intracellular mycobacteria. However one should admit that the artificial *in vitro*

situation can be very different from real *in vivo* conditions. Moreover no standardized protocols exist and the common read-out method for determination of viable mycobacteria still relies on bacterial growth analysis on plates.

4 Aims of this study

TB together with AIDS and Malaria is the major cause of death and morbidity worldwide (WHO, 2008). Co-infection with HIV leads to a dramatic course of disease and endangers public health in high incident countries (Kaufmann and McMichael, 2005). The evolvement of new MDR and XDR strains impairs the efficiency of present drug regiments (Singh, et al., 2007). In combination with the vast number of LTBI the need for a protective vaccine against adult pulmonary TB becomes obvious. Animal models fail to mimic the latent stage of infection and reliable correlates of protection against TB are still to be defined. Therefore this thesis investigated adaptive immune responses against *M. tuberculosis* in humans and tried to characterize these cellular immune responses in further detail. These issues were be addressed by three independent approaches:

1. Identification of new *M. tuberculosis* key antigens contributing to protection by comparing their T cell mediated recognition in healthy LTBI and TB patients
2. Further characterization of involved T-cell subsets in order to evaluate qualitative differences in immunity to TB among both study groups
3. Investigation of the functional properties of different *M. tuberculosis*-specific T-cell populations based on a novel flow cytometry assay

1. Adaptive cellular immune responses to TB are crucial for the outcome of infection and the help *M. tuberculosis*-specific T cells is needed to guarantee long lived protection against disease outbreak. Although unconventional antigens of *M. tuberculosis* are known, e.g. the CD1d restricted presentation of lipids or glycolipids (Schaible and Kaufmann, 2000), the most prominent immune response of $CD4^+$ as well as $CD8^+$ T cells is directed against peptides (Kawamura, 2006). Due to differences in the local cytokine/chemokine milieu, altered protein expression of the pathogen and HLA polymorphism of the host the spectrum of recognized antigens vary among individuals. Consequently identification of key antigens correlating either with protection or susceptibility would have great impact on diagnosis, monitoring of treatment and vaccination strategies. Adaptive immune responses towards *M. tuberculosis* derived proteins covering the whole bacterial life cycle (fig. 10) were studied in healthy LTBI and patients with active pulmonary TB. The antigens tested in the present study can be linked to active replication (immunodominant proteins), dormancy (DosR encoded proteins),

reactivation or resuscitation (Rpf and "Wayne antigens"). Special feature of this approach was the inclusion of latency-associated proteins of *M. tuberculosis*. We intended to document differences among both study groups to define correlates of immunity and new biomarkers for latent infection. In case of successful identification of promising candidates the immunogenic epitope within the antigen should be pinned down.

In parallel to distinct proteins fractionated *M. tuberculosis* whole cell lysate will was used to identify completely new antigens. Most studies regarding the recognition of mycobacterial antigens in TB patients and LTBI rely on known immunodominant proteins. Prominent examples are ESAT6-CFP10, TB10.4 or Ag85a/b which elicit robust IFNγ responses in infected individuals. It is important to mention that the restriction to already described antigens will limit the potential of a descriptive study and reduces its sensitivity. In order to perform unbiased clinical studies we replaced recombinant proteins by individual fractions of *M. tuberculosis* whole cell lysate.

2. To characterize the quality of the adaptive immune response to TB different parameters of *M. tuberculosis*-specific memory T cells were analyzed on a cellular level. This part of the thesis mainly focuses on the analysis of cytokine expression pattern by antigen-specific T cells in LTBI and TB. There is increasing evidence that the amount of poly cytokine producing T cells correlates with protection against viruses and other infectious diseases (Seder, et al., 2008). Hence detailed cytokine profiles were measured within different experimental setups. In this context the stimulatory effect of IL7 on poly-functional T cells was investigated since this cytokine is known to boost T_h1 T cell responses (van Roon, et al., 2003) and increased survival of *M. tuberculosis* infected mice after donation of IL7 (Maeurer, et al., 2000) has been reported, as well.

3. Although cross-sectional studies based on patients´ material may allow the identification of candidate biomarkers, the *in vivo* relevance for the outcome of infection cannot be addressed within such a clinical study. Therefore the impact of different T-cell populations was evaluated by a novel flow cytometry-based *in vitro* killing assay. It relies on the infection of human monocyte-derived macrophages with eGFP expressing mycobacteria followed by co-culture with autologous effector PBMC. The assay should allow rapid and reliable quantification of intracellular bacilli as well as evaluation of side damage to macrophages. Functional properties of different T-cell subsets were examined to enlighten the underlying processes responsible for killing of intracellular mycobacteria and the contribution of T cells.

5 Material and Methods

5.1 Material

5.1.1 Human subjects

Peripheral blood (40 ml) was obtained from 22 LTBI, recruited among health care workers at the Respiratory Diseases Clinic Heckeshorn, Department of Pneumology, HELIOS Klinikum Emil von Behring, Berlin, Germany. Diagnosis of latent *M.tuberculosis* infection was based on positive Tuberculin Skin Test (TST > 10 mm) verified by positive T-Spot TB^{TM} (cut-off 7 spots, in accordance with manufacturer's guidelines). Peripheral blood from 20 patients with clinical pulmonary tuberculosis was collected at the Respiratory Diseases Clinic Heckeshorn, Department of Pneumology, HELIOS Klinikum Emil von Behring, Berlin; at the Department of Internal Medicine/Infectious and Pulmonary Diseases, Charité, Berlin; and at the Asklepios Professional Clinic München-Gauting, Centre for Pneumology and Thorax Surgery, Munich, Germany. Blood samples were taken either before drug treatment or shortly after beginning of treatment before sputum conversion unless otherwise noted. Ten uninfected donors (TST negative) were recruited among volunteers of the Max Planck Institute for Infection Biology, Berlin, Germany. All donors were Caucasians. There was no bias in gender or age among all study groups (fig. 16). All donors gave written informed consent. The local ethics committee approved this study (205-18.1; 205-18.2; 205-18.3).

5.1.1 Proteins and peptides

Recombinant *M. tuberculosis* proteins (immunodominant antigens, dormancy-, reactivation- and resuscitation-associated proteins; Figure 10) were kindly provided by Prof. Dr T.H.M. Ottenhoff, Leiden University Medical Centre, The Netherlands (Kana, et al., 2008; Mollenkopf, et al., 2004; Park, et al., 2003; Wayne and Hayes, 1996). All proteins were expressed in, and endotoxin-free purified from, *Escherichia col*i. All peptides used for *in vitro* restimulation were purchased from JPT Peptide Technologies GmbH, Berlin, Germany in crude purity (>80%). Aliquots of 10^{-2} M stock solutions were stored at -20°C until use.

5.1.1 *M. tuberculosis* / *M. bovis* strains

The bacterial strains *M. tuberculosis* H37Rv (originally obtained from J.K. Seydel, Forschungsinstitut Borstel, Germany) and *M. tuberculosis* Beijing (RIVM No. 17919, country of isolation: Mongolia) were used for lysate generation. The recombinant eGFP expressing *M. bovis* BCG for the *in vitro* infection assays was kindly provided by Dr. Nathlie Winter, Mycobacterial Genetics Unit, Institut Pasteur, France. In brief *M bovis* BCG Pasteur 1173P2 was transformed with Ms6-derived integrative vectors11 expressing the *Aequoria victoria egfp* gene.

5.1.1 Equipment

Flow cytometer FACSCanto™II and LSRII	Becton Dickinson
ELISA-reader SpectraMAX190	Molecular Devices
Bio-Plex™ System	BioRad
MultiScreen Filtration System	Millipore
FAST-Prep™ System Bio 101	Savant
Ultra-sound bath Sonorex RK100	Bandelin
CO_2-Incubator	Binder
Incubator	Memmert
Microcentrifuge Biofuge fresco	Heraeus
Megafuge 1.0R (sepatech#2704 rotor)	Heraeas
Multifuge 4KR (LH4000 rotor)	Heraeus
Biological Safety Cabinets Class II (NUAIRE and HeraSafe)	Heraeus
Vortex MS1 Minishaker	IKA Labortechnik
Electronic pipetter Pipetboy acu	Integra Biosciences
pH-meter 761 calimatic	Knick
Lab-Shaker	Lab-Therm
Microscopes	Leica
Cell counting chamber Neubauer improved	Brand
Scales	Sartorius
Water purifier	Millipore

5.1.1 Kits

IFNγ / TNFα / IL2 Secretion Assays	Miltenyi
CytoFix/CytoPerm Kit	BD
Human IFNγ ELISA	BD
Human TNFα ELISA	BD
Bio-Plex™ human T_h1 cytokine Kit	BioRad
Bio-Plex™ human IL17 cytokine Kit	BioRad

5.1.2 Antibodies

Tab. 1: Fluorchrome-labeled antibodies used in this thesis.

Antibody specificity	Fluorochrome	Clon	Order number	Supplier
CCR7	PE	3D12	552176	BD
CD3	APC-Cy7	SK7	557832	BD
	FITC	UCHT1	555332	BD
	Pacific Blue	UCHT1	558120	BD
	PerCP	SK7	345766	BD
CD4	Alexa 700	OKT-4	56-0048	eBioscience
	APC	RPA-T4	555349	BD
	APC-Cy7	SK3	341115	BD
	AmCyan	SK3	339187	BD
	FITC	SK4	345768	BD
	Pe-Cy7	SK3	557852	BD
CD8	PE-Cy7	SK1	335822	BD
	PerCP-Cy5.5	SK1	341050	BD
	PerCP	SK1	345774	BD
	APC-Cy7	SK1	557834	BD
CD14	APC	M5E2	555399	BD
	PE	M5E3	555398	BD
CD154 (CD40L)	PE	TRAP1	555700	BD
	PE-Cy5	TRAP1	555701	BD
CD45RA	APC	HI100	550855	BD
CD45RO	PE	UCHL1	347967	BD
	PE-Cy5	UCHL1	555494	BD
	PE-Cy7	UCHL1	337168	BD
CD56	PE	MY31	345810	BD
CD62L	APC	145	120-002-048	Miltenyi
	APC	Dreg 56	559772	BD
	APC-Alexa 750	Dreg 56	27-0629-73	eBioscience
	APC-Alexa 750	Dreg 56	27-0629	eBioscience
CD69	APC	FN50	555533	BD
CD107a (LAMP-	FITC	H4A3	555800	BD
CD127a (IL7R)	APC	KEN06	FAB306A	R&D
CXCR3	APC	#49801	FAB160A	R&D
CXCR5	PE	#51505	FAB190P	R&D
CXCR6	APC	#56811	FAB699A	R&D
HLA-A02	FITC	BB7.2	551285	BD
IFNg	FITC	4S.B3	554551	BD
	APC	25723.11	341117	BD
IL2	FITC	MQ1-17H12	559361	BD
	FITC	MQ1-17H12	554565	BD
	PE	MQ1-17H12	559334	BD
IL3	PE	BVD3-1F9	554676	BD
IL4	PE	MP4-25D2	554485	BD
	PE	8D4-8	559333	BD
TNF	FITC	64.011.111	340511	BD
	Pacific Blue	MAb11	57-7349-73	eBioscience
	Pacific Blue	MAb11	57-7349	eBioscience
GMCSF	PE	BVD2-21C11	554507	BD
IL-17	PE	eBio64DEC17	12 7179	eBioscience
IL12 (p70)	PE	20C2	557020	BD
IL12 (p40/p70)	APC	C11.5	554576	BD
IL-13	PE	JES10-5A2	559328	BD
IP-10	PE	6D4/D6/G2	555049	BD

All conjugated antibodies for FACS analysis were purchased by Becton Dickenson (BD), eBioscience and Miltenyi, respectively.

5.1.3 Other material

Addresses and contact information for suppliers are listed in the appendix. Standard laboratory chemicals used to prepare buffers, staining solutions and the like, were purchased from Sigma, Merck or Roth in *per analysis* quality.

PARAFILM®	Brand
Cell strainer (40µm)	BD
Syringes	B.Braun
Steritop Sterile Filters	Millipore
Millex Syringe Driven Filter Units	Millipore
Sterile filters and membranes	Schleicher & Schüll
CombiTips	Eppendorf
Disposable Scalpels	B.Braun
6-well / 24-well / 48-well / 96-well Flat Bottom Plates	NUNC
96-well Round Bottom / V Bottom Plates	NUNC
12-well / 96-well Ultra Low Attachment Plates	Corning
ELISA-plates Immuno Maxi-Sorp	Nunc
CryoTubes 1.8ml	NUNC
Filter plates for Bio-Plex™ MultiScreen®-BV	Millipore
FACS Tubes 5.0ml	Sarstedt
Cluster Tubes 1,8ml	Thermo Fischer
Disposable Transfer Pipette	BD
Steripette (5.0ml/10ml/25ml/50ml)	Costar
Disposable Inoculating Loops	NUNC
Bradford Assay reagent	BioRad
Bovine Serum Albumin (BSA)	Serva
Brefeldin A	Invitrogen
Staphylococcus enterotoxin B (SEB)	Invitrogen
Phytohemaglutinin (PHA)	Invitrogen
Recombinant cytokines (IL2, IL7, IFNγ, TNFα, GM-CSF)	all Invitrogen
Tuberculin PPD Batch RT50	Satents Serum Institute
Streptavidin-PE (1mg/ml)	Invitrogen
UltraPURE Water	Invitrogen
Plasticware	Corning, Sarstedt or TPP
Glassware	Schott

5.1.4 Buffers and solutions

Solutions were made up in H_2O prepared with a Millipore water purifier, unless otherwise noted. Where indicated (*), solutions were sterilised by autoclaving for 25min at 121°C, or filter-sterilised through a 0.2µm membrane.

Cell culture media

FCS-RPMI medium*:	10% (v/v) FCS 1.0mM L-glutamine 100U/ml penicillin 100µg/ml streptomycin 10mM HEPES Buffer
HS-RPMI medium*:	10%(v/v) heat inactivated human AB Serum 1.0mM L-glutamine 100U/ml penicillin 100µg/ml streptomycin 10mM HEPES Buffer

Bacterial culture media

Middlebrook 7H9 broth*:	4.7g DifcoTM Middlebrook 7H9 powder 2ml glycerol 900ml water
Added after autoclaving:	100ml BBL™ Middlebrook 10% (v/v) ADC enrichment 0.05% (v/v) Tween80
7H11-agar plates*:	21g Difco™ Mycobacteria 7H11 agar 5ml glycerol 900ml water
Added after autoclaving:	100ml BBL™ Middlebrook OADC enrichment

Optional: 50mg/l kanamycine were added after cooling.
(50g/l stock solutions of kanamycine were stored at –20°C)

PBS/Tween (PBST)*:	0.5% (v/v) Tween80 in PBS

Buffers for ELISA, Bio-Plex™ and flow cytometry

Phosphate buffered saline (PBS):	8g NaCl 0.2g KCl 0.2g KH_3PO_4 1.3g Na_2HPO_4
ELISA coating buffer:	100mM Na_2CO_3 100mM $NaHCO_3$ titrated to pH9.6
ELISA wash buffer:	0.1% (w/v) BSA 0.05% (v/v) Tween 20 in PBS
ELISA blocking buffer:	5% (w/v) BSA in PBS
ELISA stop solution:	3M NaOH
Bio-Plex assay buffer A:	1% (w/v) BSA 0.05% (v/v) Tween 20 in PBS
Bio-Plex wash buffer A:	0.5% (w/v) BSA 0.05% (v/v) Tween 20 in PBS
FACS buffer (PBS/B)*:	10% FCS in PBS

5.1.5 Software

Tables, calculations, statistic and graphics

GraphPad Prism 4.0 GraphPad Software

Excel 2007 Microsoft

Photoshop Adobe Systems

Flow cytometric analysis

FCS Express 3.0 De Novo Software

FACSDiva v5.0.2 BD

FACS data Analyser (v0.9.8) Christian Köberle

Bio-Plex analysis

Bio-Plex Manager 4.1.1 Bio-Rad

Text

Word 2007 Microsoft

References

Endnote X Thomson

5.1.6 Web resources

Sequence information on *M .tuberculosis* genes:
http://genolist.pasteur.fr/TubercuList/

Information on cytokines and chemokines:
http://www.copewithcytokines.de/cope.cgi

5.2 Methods

5.2.1 Cell culture for intracellular cytokine analysis

PBMC were isolated by density centrifugation (Biocoll, Biochrom) following manufacturer's instructions. Cells were resuspended in RPMI-1640 (GIBCO, Invitrogen) supplemented with 10% AB Rh-positive heat-inactivated human serum (Sigma-Aldrich), 100U/ml penicillin, 100µg/ml streptomycin (PAA), 1mM L-glutamine (PAA) and 10mM HEPES (PAA). 2×10^5 PBMC per well were seeded in 96-well round bottom plates (NUNC) in 200µL medium. Cells were cultured at 37°C / 5% CO_2 with medium alone or together with 1µg/ml Staphylococcus enterotoxin B (SEB, Sigma-Aldrich), 10µg/ml purified protein derivative (PPD) from *M. tuberculosis* (Statens Serum Institute), 5µg/ml recombinant *M. tuberculosis* proteins (fig. 10) either for 16h or 7d. In order to identify the immunogenic epitope of Rv3407 we designed overlapping peptide pools and tested these pools in the 7d two rounds of restimulation assay. Each 15-mer peptide was added at a final concentration of 5µg/ml. The pools were designed as matrix pools as described before (Maecker, et al., 2001). In brief, 15-mer peptides with a shift of 3 amino acids along the primary sequence represent the whole protein and allow mapping of $CD4^+$ as well as $CD8^+$ T-cell responses. For a complete list of all tested peptides see figure 19. A second restimulation with the respective antigen was performed for the long-term assay at day 6. In both cases, 10µg/ml of the secretion inhibitor brefeldin A (Sigma-Aldrich) was added 12h before analysis to avoid release of cytokines by the Golgi apparatus. Subsequently, cells were fixed and permeabilized using cytofix/cytoperm

(100 µl) (BD Biosciences) for 30min at 4°C. Treatment with Cytoperm/wash (150µl) (BD Biosciences) was applied twice before staining with fluorochrome-labeled antibody mixtures (mAb). Staining antibodies were used in different combination and a complete list of FACS antibodies is given as figure 1. After staining for 45min at 4°C, cells were washed twice in Cytoperm/wash, resuspended in phosphate-buffered saline (PBS, Gibco) containing 10% fetal calf serum (FCS, Invitrogen) and analyzed using a FACS-LSRII (BD Biosciences).

5.2.2 Cytokine analysis in the culture supernatant by ELISA

Due to the accumulation of IFNγ and TNFα in the supernatant during prolonged *in vitro* stimulation the initial number of PBMC per well for ELISA has been reduced down to $5*10^4$ cells in 200µL medium. Following the described protocol (see 5.2.2 *Cell culture and intracellular cytokine analysis*) cells were stimulated at day 0 and day 6 under the same conditions. In contrast to the former approach no secretion inhibitor was added and 110µL of cell culture supernatant was harvested at day 7. To evaluate the impact of the doubled restimulation, supernatants were also harvested early at day 6 for some individuals. The remaining cells were used for the proliferation assay. Commercially available IFNγ and TNFα ELISA´s (*BD Biosciences*) were performed according to manufacturer's guidelines. In brief, 96 well flat bottom plates (NUNC) were coated overnight at 4°C with a primary catch mAb directed against the respective cytokine. After blocking with 10% FCS/PBS either 50µL of cell culture supernatant or different dilutions of the reference cytokine standard was applied. After extensive washing wells were incubated with a biotinylated secondary mAb together with Streptavidin-horseradish peroxidase. Finally tetramethylbenzidine substrate solution (*BioRad*) was added and the enzymatic reaction was stopped with 1M phosphoric acid. Plates were analysed by measuring extinction at 450nm using an ELISA plate reader (*Molecular Devices*).

5.2.3 Proliferation analysis by [^{3}H]-Thymidine incorporation

After harvesting supernatants for ELISA 50µL medium containing ^{3}H-labeled thymidinee (1,25 µCi) was added and cells were incubated at 37°C for 6h. After the incorporation period cells were frozen at -20°C thawed and transferred to 96 well Unifilter plates using a harvester (both *PerkinElmer*). Plates were then dried at 50°C for 30min and 40µL of Liquid

Scintillation Cocktail (MicrosCint, *PerkinElmer*) was added. The sealed plates were finally analyzed by using a TopCount reader (*PerkinElmer*).

5.2.4 *In vitro* killing assay

Buffy coats from the blood donor service of the Charité University Hospital, Berlin were used for isolation of PBMC in high numbers as already described.

Macrophage generation and infection with mycobacteria: Monocytes were separated by adherence to plastic by incubation for 12h at 37°C and 5% CO_2 in presence of 50U/ml recombinant GM-CSF (Invitrogen) in RPMI-1640 (GIBCO, Invitrogen) supplemented with 10% AB Rh-positive heat-inactivated human serum (Sigma-Aldrich), 1mM L-glutamine (PAA), 100U/ml penicillin, 100µg/ml streptomycin (PAA) and 10mM HEPES (PAA). To detach adherent monocytes from the bottom of 75cm^2 cell culture flasks they were washed with PBS (Gibco) and treated with 2mM EDTA. Residual cells were mechanically detached by a cell scraper if needed. Monocytes were then centrifuged and 7,5*10^4 cells were seeded into 96 well round bottom ultra-low-attachment plates (ULA plates, Corning) in 100µL RPMI-1640 supplemented with 10% human serum (see above). To allow differentiation of monocytes to macrophages the cells were incubated at 37°C and 5% CO_2 for further 4d. For infection of macropahges with mycobacteria eGFP expressing BCG were grown in Middelbrook 7H9-broth medium supplemented with 10% ADC enrichment at 37°C with shaking until bacterial growth reached an OD600=0.7, corresponding to a cell density of approx. 10^{8}cells/ml. These mid-logarithmic cultures were harvested by centrifugation, washed twice with PBS and homogenized by transfer through a 26G needle. BCG were counted by using a Neubauer Improved Chamber and applied at an MOI 5 and 20. Infection took place at 37°C, 5% CO_2 and moderate shaking for 4h. After infection medium was removed and marcophages were washed four times with 200µl preheated RPMI-1640 (GIBCO, Invitrogen) supplemented with 10% AB Rh-positive heat-inactivated human serum (Sigma-Aldrich), 1 mM L-glutamine (PAA) and 10 mM HEPES (PAA). After each washing step plates were centrifuged and the supernatant was flipped on absorbing paper. Finally 100µL medium was added.

Generation of autologous effector PBMC: PBMC were incubated at a final concentration of 1*10^{6}cells/ml in upright 20cm^2 cell culture flasks 37°C and 5% CO_2 in RPMI-1640 (GIBCO, Invitrogen) supplemented with 10% AB Rh-positive heat-inactivated human serum (Sigma-Aldrich), 1 mM L-glutamine (PAA), 100 U/ml penicillin, 100 µg/ml streptomycin (PAA) and

10 mM HEPES (PAA). To promote their effector function for the later assay *M. tuberculosis* whole cell lysate (10µg/ml), different concentrations of recombinant IL2 (Invitrogen), recombinant GM-CSF (Invitrogen), recombinant IFNγ (Invitrogen) or phytohemaglutinin (10 µg/ml) (PHA, Invitrogen) were added. After the first 24h 10U/ml recombinant IL2 was given to promote mid-term survival of PBMC. After additional 4d of incubation cells were washed with PBS and resuspended in 100µL RPMI-1640 (GIBCO, Invitrogen) supplemented with 10% AB Rh-positive heat-inactivated human serum (Sigma-Aldrich), 1 mM L-glutamine (PAA), and 10 mM HEPES (PAA). For co-culture of infected macrophages and autologous PBMC, effector cells were adjusted at different effector:target ratios and incubated at 37°C and 5%CO_2 for 16-36h.

For FACS analysis palates were centrifuged and surface staining for prominent lineage marker was performed in 100µl PBS containing 10% FCS and 2mM EDTA. After incubation at 4°C for 30min cells were washed twice with 10% FCS / PBS, resuspendend in 100µl 10% FCS / PBS and analysed using a LSR II (BD Bioscience). If CFU were performed in parallel plates were centrifuged and cell lysis was achieved by addition of 0.5% EDTA in PBS. Serial dilutions were performed in 0.5% Tween / PBS and 50µl volume were plated on 7H11 agar plates (diameter 10cm) which were sealed with PARAFILM® and wrapped in aluminium foil. After three weeks of incubation at 37°C cfu were counted.

5.2.5 Bio-Plex Assay

The Bio-Plex™ Human Cytokine T_h1 panel and IL-17 Assay were performed according to the manufacturer's instructions (BioRad) with some slight modifications. Only 0.5µl beads in 50µl Assay buffer A, 0.15µl (50x concentrated) or 0.075µl (100x concentrated) of the detection antibodies in 25µl Assay buffer A and 0.2µl Streptavidin-PE (1mg/ml) in 50µl Assay buffer A per well were used. Beads as well as detection antibodies from all kits were mixed together in order to analyze all cytokines in parallel within the same sample.

5.2.6 Lysate generation

M. tuberculosis was grown under BSL3 conditions in Middelbrook 7H9-broth medium at 37°C with shaking until bacterial growth reached an OD600=0.7, corresponding to a cell density of approx. 10^8cells/ml. These mid-logarithmic cultures were harvested by centrifugation, washed twice with PBS and lysed using the FastPrep™ homogenizer

technology (Savant). Following the manufactures' protocol for protein isolation Matrix B columns were used to disrupt bacilli by two 45 s pulses at 6,000 x *g*. The lysate was centrifuged and the debris-free supernatant was collected. The supernatant mainly contains *M. tuberculosis* cytosolic components including the fraction of cytosolic proteins. Finally the lysate was applied to a steril filter (20µm) and the protein yield was determined by Bradford assay.

5.2.7 Bradford assay

Bradford Assay was used to determine the total protein concentration of *M. tuberculosis* lysate and separate Beijing fractions after gel filtration. Basically the absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 (BioRad) shifts from 465nm to 595nm when binding to protein occurs. Serial dilution of BSA was used as standard for absolute determination of the protein yield. The absorbance was measured at 600nm using an ELISA plate reader (*Molecular Devices*).

5.2.8 Gel Filtration

To separate the cytosolic proteins according to their particular molecular size the Beijing lysate was applied to an Amersham Superose 12 gel filtration column with a fraction range from 1kDa to 300kDa. Basically smaller molecules show a more intense interaction with the gel matrix than larger molecules. Consequently it needs more time to elute them from the column and by subsequent collection of the flow (PBS, Gibco) molecules of different sizes can be separated. The chromatography of each fraction can be used to determine the absorption at 280nm. Since this is the characteristic absorption maximum for aromatic amino acids (tryptophan, tyrosine, phenylalanine) it reflects the total protein load of the single fraction.

5.2.9 Analysis procedures and statistics

For analyses memory T helper cells (we have previously identified this T-cell subpopulation as the major producers of cytokines in this assay (Mueller, et al., 2008)) were gated according to size, granularity, CD3, CD4, and CD45RO expression (fig. 11). Absolute numbers and proportions of cytokine expressing cells were determined. The Mann-Whitney U-test was used to determine significant differences between study groups and T-cell subpopulations. For

the individual comparison (fig. 15) a paired t-test was used. Nominal two-sided p-values are given and considered significant if $P < 0.03$ (*); $P < 0.01$ (**); $P < 0.0001$ (***).

5.2.10 Discrimination analysis

The discriminatory power for classifying TB patients and healthy LTBI was investigated using random forest analysis (Breiman, 2001). The classification was based on the proportion of IFNγ expressing $CD4^+$, $CD45RO^+$ T cells against of 14 selected stimuli (i.e. SEB, PPD, ESAT-6_CFP10, Rv0569, Rv1733c, Rv1734, Rv2003, Rv2005c, Rv2006, Rv0140, Rv1009, Rv1884c, Rv2450c and Rv3407). We determined the relative feature importance for discrimination using a leave-1-out cross validation for all possible combinations of genes and assessed the proportion of correctly classified patients in the left-out group.

6 Results

Working hypothesis of this thesis is the assumption that differences in the adaptive immune response either lead to long-lived protection against secondary tuberculosis or to an impaired containment of the pathogen with reactivation of disease. In order to identify such differences the adaptive immune response against *M. tuberculosis* will be compared between two study groups; on the one hand HIV negative patients with active pulmonary tuberculosis (the immune system failed to contain the pathogen) and on the other hand healthy donors with long lasting latent *M. tuberculosis* infection (proper containment).

Possible distinctions between both sub-groups will be addressed by the analysis of *M. tuberculosis* antigens recognized and by cellular cytokine expression patterns of *M. tuberculosis*-specific $CD4^+$ T cells. Finally functional properties of different T cell subsets will be studied by a newly developed *in vitro* assay based on monocyte-derived macrophages infected with mycobacteria. General focus is on the identification of key factors relevant for the outcome of infection.

6.1 Identification of relevant *M. tuberculosis* antigens

As already mentioned the presentation of *M. tuberculosis* derived antigens by APC and the generation of adaptive immune responses are crucial for robust and long lived immunity to TB (see *3.2.2 The immune responses to M. tuberculosis*). Although unconventional antigens of *M. tuberculosis* are known, e.g. CD1d restricted lipids or glycolipids (Schaible and Kaufmann, 2000), the most prominent immune response of $CD4^+$ as well as $CD8^+$ T cells is directed against peptides (Kawamura, 2006). Since the entity of *M. tuberculosis* antigens represent “molecular targets” mediating recognition of infected host cells the identification of relevant protein-antigens will rely on two parallel approaches:

i) Fractionated *M. tuberculosis* lysate will be used for restimulation of PBMC from LTBI and TB patients in order to characterize completely new antigens.
ii) The adaptive immune response towards a panel of recombinant latency-associated proteins of *M. tuberculosis* will be analyzed in healthy LTBI and TB patients.

6.1.1 Fractionated *M. tuberculosis* lysate

Most studies regarding the antigen recognition of TB patients and LTBI depend on known immunodominant proteins. Prominent examples are ESAT6, CFP10, TB10.4 or Antigen85a/b which elicit robust IFNγ responses in infected individuals. The restriction to known antigens limits the potential of a descriptive study. In order to perform unbiased clinical studies we intend to replace recombinant proteins used as antigens by distinct fractions of *M. tuberculosis* whole cell lysate. Instead of the commonly used lab strain H37Rv of *M. tuberculosis* we decided to perform this experiment with a hyper-virulent clinical isolate called Beijing (Wong, et al., 2007). This should mirror the real clinical situation in high endemic areas. In a first approach to separate the cytosolic proteins according to their particular molecular size a crude whole cell lysate of the Beijing strain was applied to a gel filtration column. The chromatography of this gel filtration is shown in figure 6.

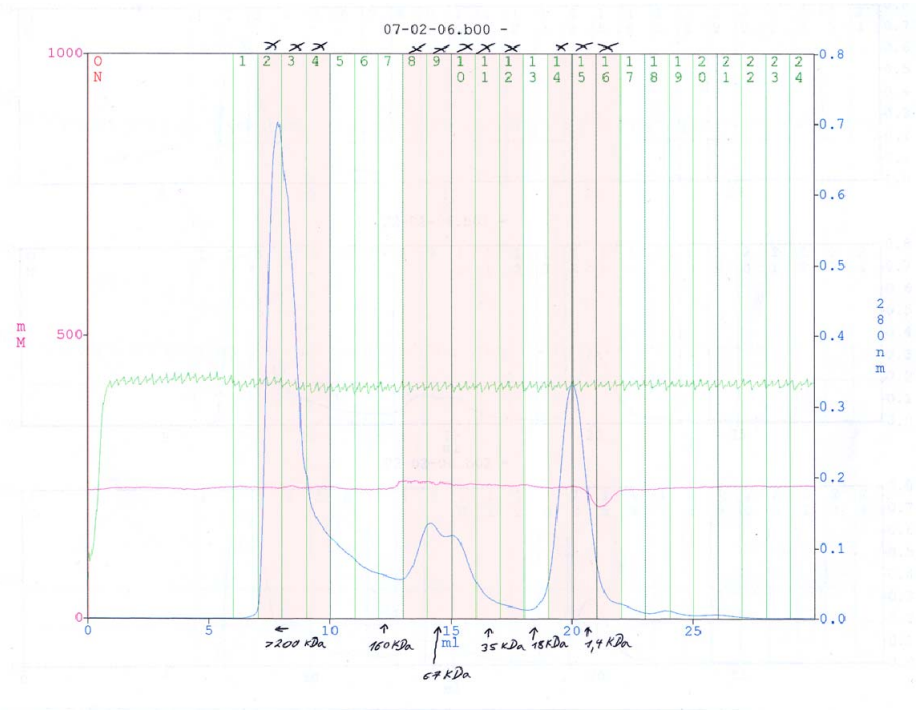


Fig. 6: Gel filtration of *M. tuberculosis* whole cell lysate.

Cytosolic whole cell lysate of the hypervirulent Beijing strain was applied to an Amersham Superose 12 column with a fraction range from 1 kDa to 300kDa. The x-axis shows the flow volume in ml and the typical size of molecules which can be eluted with that volume (arrows). The blue curve of the chromatography indicates absorption at 280nm. The number of each separate fraction is depicted in green and red-labeled fractions were selected for restimulation assays due to high protein yield.

The overall flow volume is indicated in ml at the bottom of the graph as well as the typical molecular size of "reference molecules" applied to the same column. The blue curve represents absorption at 280nm. Since this is the characteristic absorption maximum for aromatic amino acids (tryptophan, tyrosine, phenylalanine) it reflects the protein yield. At the top the number of each individual fraction is indicated and protein-rich fractions selected for restimulation assays are labeled in red. For each fraction the individual protein concentration was determined by Bradford assay.

In order to evaluate the reproducibility of this method two independent batches of Beijing lysate were applied to the same gel filtration column. For both batches the chromatography is shown in figure 7. The characteristic absorption profile at 280nm (blue curve) is identical for both samples. Since this is no proper way of "quality control", the problem of reproducibility was avoided by using the same batch of Beijing fractions for the following study.

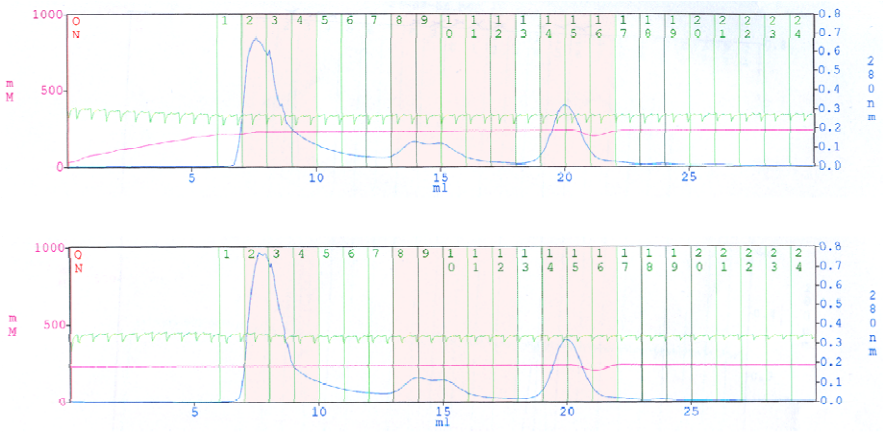


Fig. 7: Gel filtration of two different batches of *M. tuberculosis* lysates.

Cytosolic whole cell lysates of two different batches of the Beijing strain were applied to the same column (as described before). The characteristic absorption profile at 280nm is indicated by the blue curve.

To measure the adaptive immune response towards the Beijing fractions an *in vitro* restimulation assay was used. In brief PBMC from healthy LTBI (T-Spot TB positive), patients with active pulmonary tuberculosis and uninfected controls (TST negative) were incubated for 16h with each separate fraction. 12h prior to analysis the secretion inhibitor brefeldin A was added to inhibit cytokine release. Finally cells were fixed and stained for intracellular cytokines as well as phenotypic markers. In this assay only memory T cells which were already primed by the same antigen will express characteristic effector cytokines. FACS analysis focused on $CD3^+$ $CD4^+$ lymphocytes for the detection of antigen-specific T-helper cell responses. The analysis includes the specific activation marker CD154 to avoid the detection of unspecific activation (TLR or bystander activation) which could be due to non-protein components within the lysate. $CD3^+$ $CD4^+$ T cells were then analyzed for the expression of IFNγ, TNFα, IL2 and GM-CSF. Parallel analysis of $CD8^+$ T cells revealed no detectable cytokine expression (IFNγ, TNFα, IL2, GM-CSF) in any case. 16h *in vitro* restimulation was performed for two TST negative controls, four LTBI and five patients with active TB before/within the first week of treatment (fig. 8).

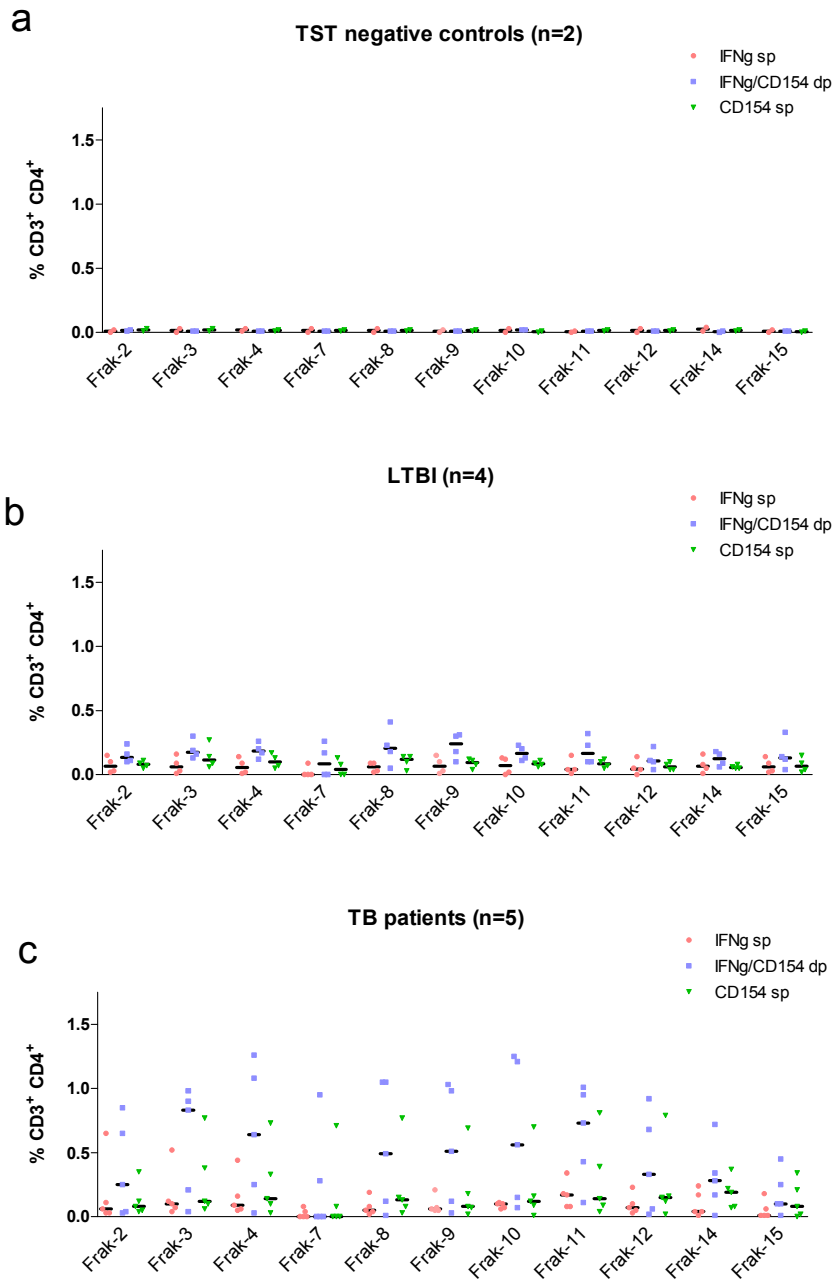


Fig. 8: ICS after 16h of *in vitro* incubation revealed T-cell mediated recognition of Beijing fractions in LTBI and TB patients.

Fractionated Beijing whole cell lysate (10µg/ml) was used to restimulate $5*10^5$ PBMC in 200µl RPMI containing 10% human serum. After 4h of incubation the secretion inhibitor brefeldin A was added at a concentration of 15µg/ml. Following additional 14h of incubation at 37° and 5 % CO_2 cells were fixed, permeabilized, stained and analyzed by 6-colour flow cytometry. Results are shown for **a)** two non-*M. tuberculosis* infected donors **b)** four latently infected donors and **c)** five patients with active pulmonary TB. Background values of unstimulated controls were subtracted during data analysis. Numbers of Beijing fractions are indicated at the x-axis and percentage of $CD3^+$ $CD4^+$ T cells is shown on the y-axis. For each fraction and donor the amount of IFNγ single positive cells (sp, red dots), IFNγ/CD154 double positive cells (dp, blue squares) and CD154 sp cells (green triangles) is depicted.

Data analysis was done for $CD3^+$ $CD4^+$ T cells and discriminated between IFNγ/CD154 double positive (dp), IFNγ single positive (sp) and CD154 sp cells. Individual background values for unstimulated medium controls were always subtracted. For uninfected donors IFNγ as well as CD154 expression was not detectable. Therefore cytokine expression by LTBI or TB patients can be considered antigen-specific. All four LTBI showed detectable proportions of IFNγ/CD154 dp cells for the vast majority of Beijing fractions. In most cases slightly elevated levels of CD154 sp cells were measured. IFNγ sp cells were only detected at low frequencies underlining the value of CD154 as a marker for antigen-specific activation of $CD4^+$ T cells. Regarding the quantity and the quality of the immune response no differences between the separate fractions could be observed by using this assay. For LTBI $CD4^+$ T-cell response seems to be independent from the particular Beijing fraction. Consequently we failed to identify specific fractions which elicit more prominent T-cell responses in LTBI.

In patients with active TB the frequencies of activated $CD4^+$ T cells were generally higher compared to LTBI. Similar to LTBI the highest frequencies were detected for IFNγ/CD154 dp cells, but also detectable amounts of CD154 sp cells were measured. In contrast to LTBI in a few cases IFNγ was detected without co-expression of CD154. This could be explained by bystander activation due to high cytokine levels before addition of the secretion inhibitor. An additional observation is the broader range of T-cell frequencies for TB patients compared to LTBI (higher variability). In summary, no consistent pattern regarding the recognition of Beijing fractions could be found for the five TB patients.

For a single patient we had the possibility to collect peripheral blood at the beginning of treatment and four weeks later after sputum conversion (fig. 9). Before therapy the TB patient showed a pronounced IFNγ response mediated by $CD4^+$ T cells towards some of the Beijing fractions (Frak8; 9; 10; 11). The analysis at the later time point revealed a general increase in $CD4^+$ T-cell responses which were now directed against the majority of the Beijing fractions. 11 out of 14 fractions now elicited high frequencies of $IFN\gamma^+$ T cells. Moreover the cellular activation/cytokine pattern seems to be altered since the absolute increase in T-cell frequencies also caused an increase in the relative amount of CD154 sp cells (orange bar in figure 9a and 9b). The cellular cytokine secretion pattern in context of these Beijing fractions will be discussed in further detail later in this thesis.

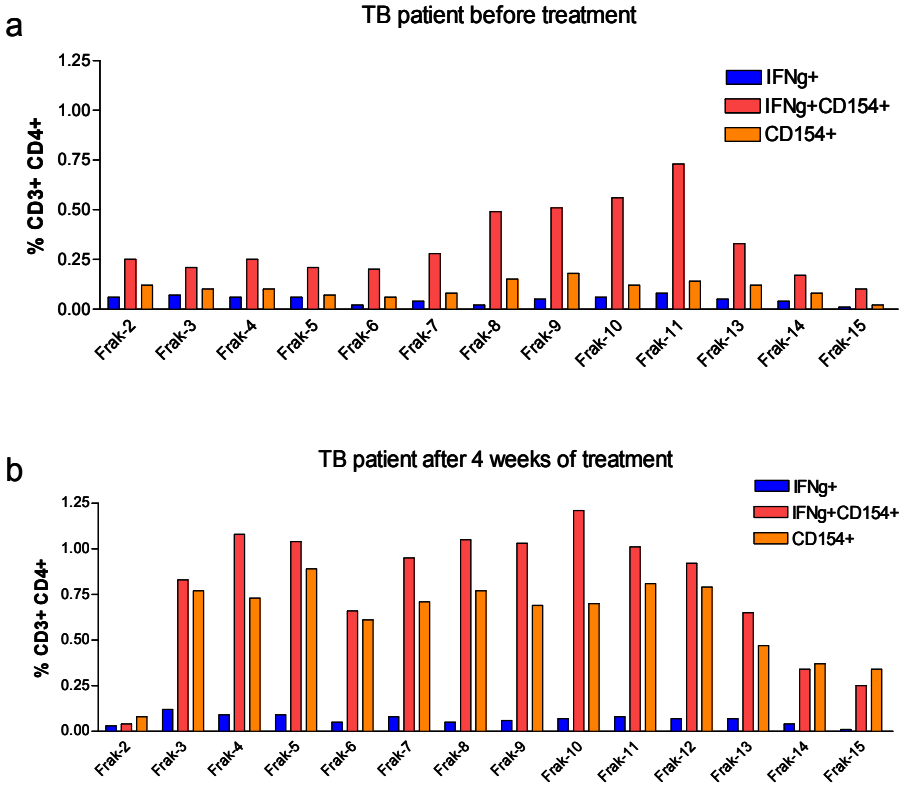


Fig. 9: Increased proportions of specifically activated CD4$^+$ T cells after 4 weeks of treatment measured by ICS.

Fractionated Beijing whole cell lysate was used to restimulate PBMC from a patient with active pulmonary TB as already described. Peripheral blood was drawn **a)** before beginning of treatment and **b)** after four weeks of first line antibiotic treatment (including isoniazid, rifampicine, pyrazinamide and ethambutol). Background values of unstimulated controls were subtracted during data analysis. Numbers of Beijing fractions are indicated at the x-axis and percentage of CD3$^+$ CD4$^+$ T cells is shown on the y-axis. For each fraction the amount of IFNγ sp cells (blue bars), IFNγ/CD154 dp cells (red bars) and CD154 sp cells (orange bars) is depicted.

6.2 Latency-associated antigens

This parallel approach is focused on the detection of adaptive immune responses towards recombinant *M. tuberculosis* proteins associated with dormancy, reactivation or resuscitation (fig.10). For comprehensibility reasons these proteins will be termed "latency-associated" from now on since they are unregulated by *M. tuberculosis* during the latent stage of disease. Despite the efforts to identify new biomarkers for latent *M. tuberculosis* infection (correlate of protection) it is worth to speculate that T-cell responses towards latency-associated antigens actively contribute to immunity to TB (correlate of immunity). This is a first step to verify the

hypothesis that T-cell responses towards latency-associated antigens are essential for the outcome of mycobacterial infections.

No.	Name	Category
1.01	ESAT6_CFP-10	Immunodominant proteins
1.02	TB10.4	
1.03	Rv3019c	
1.04	Ag85A	
1.05	Ag85B	
1.06	Hsp65	
2.01	Rv0081	DosR regulon encoded proteins
2.02	Rv0569	
2.03	Rv0573c	
2.04	Rv1733c	
2.05	Rv1734	
2.06	Rv1735c	
2.07	Rv1736c_C term	
2.08	Rv1996	
2.09	Rv1997_C term	
2.10	Rv1997_N term	
2.11	Rv1998	
2.12	Rv2003	
2.13	Rv2005c	
2.14	Rv2006	
2.15	Rv2032	
2.16	Rv2623	
2.17	Rv2624c	
2.18	Rv2625c	
2.19	Rv2628	
2.20	Rv3126c	
2.21	Rv3129	
2.22	Rv3133c	
3.01	Rv0140	Proteins associated with reactivation
3.02	Rv1115	
4.01	Rv0867c	Resuscitation promoting factors
4.02	Rv1009	
4.03	Rv1884c	
4.04	Rv2450c	
5.01	Rv3407	Reactivation

Fig. 10: Complete list of recombinant *M. tuberculosis* proteins tested.

Recombinant proteins of *M. tuberculosis* tested in *in vitro* restimulation assays. Known immunodominant proteins (1.xx) as well as DosR regulon encoded proteins (2.xx), resuscitation promoting factors (4.xx) and proteins associated with reactivation (3.xx + 5.xx) were included.

The panel of tested *M. tuberculosis* proteins represent the complete life cycle of the pathogen (fig. 5) with a focus on the latent stage of disease. The list of 35 recombinant *M. tuberculosis* proteins includes known immunodominant antigens (1.xx), DosR encoded proteins (2.xx) and proteins associated with resuscitation or reactivation (3.xx, 4.xx, 5.xx). More details about the role of these proteins can be found in the *Introduction* of this thesis.

6.2.1 Assay optimization and candidate identification

In a first step 16h *in vitro* restimulation was used as already described. This method is based on short term incubation of PBMC in medium supplemented with proteins / antigens of interest. To inhibit the release of cytokines a secretion inhibitor, e.g. brefeldin A, was added to the cells 12h prior to analysis. Finally, PBMC were fixed and stained for phenotypic markers and intracellular cytokines. Due to the short-term restimulation only cells which were already primed in the host (i.e. memory cells) will express cytokines and can be detected by flow cytometry. To verify that the responses are carried out by specific memory T cells an unstimulated medium control as well as the memory marker CD45RO is included.

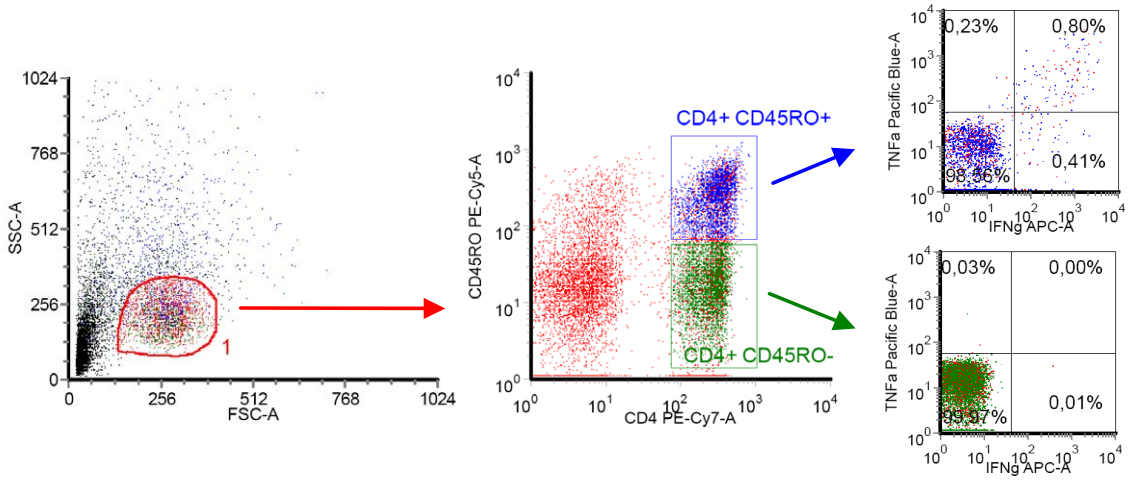


Fig. 11: Gating strategy for FACS analysis after *in vitro* restimulation and ICS.

$2*10^5$ PBMC from a LTBI were restimulated with PPD from *M. tuberculosis* (10µg/ml) in 200µl medium containing human serum for 16h. After 4h the secretion inhibitor brefeldin A was added (15µg/ml) and cells were finally fixed and stained. First lymphocytes were gated due to characteristic size (forward scatter, FSC) and granularity (sideward scatter, SSC). In a second gating step T-helper cells were selected by the expression of CD4. Next memory T cells ($CD4^+$ $CD45RO^+$) were analyzed independent form naïve T cells ($CD4^+$ $CD45RO^-$) for the expression of IFNγ and TNFα.

Post-acquisition analysis of FACS data is demonstrated in figure 11 for a latently infected donor and 16h restimulation with purified protein derivative (PPD) of *M. tuberculosis*. Lymphocytes are selected by their characteristic size (FSC) and granularity (SSC). Only $CD4^+$ T cells were chosen for further analysis and studied separately according to their expression of CD45RO. Finally the expression of IFNγ and TNFα was measured on a cellular level. For individual experiments GM-CSF, IL2, CCL4 or CD154 were included to analysis.

The expression of tested cytokines was restricted to $CD45RO^+$ $CD4^+$ T cells, which is representative for all experiments performed with PPD, recombinant proteins or synthetic

peptides. Detectable expression of cytokines for $CD45RO^-$ and $CD8^+$ T cells could only be observed after incubation with the superantigen staphylococcus enterotoxin B (SEB), which serves as a positive control in this study. Moreover a strong correlation of the expression of IFNγ and TNFα could be documented. The proportion of IFNγ expressing T cells is shown in all the following figures. The vast majority of cytokine expressing $CD4^+$ T cells were also positive for the antigen-specific activation marker CD154 (data not shown).

The first step was to verify the recognition of latency-associated proteins of *M. tuberculosis* in LTBI before comparing these responses to patients with active TB. The results of ICS and FACS analysis for 11 healthy latently infected donors are shown in figure 12. Diagnosis of LTBI is based on a positive TST skin test verified by T-Spot TB (for details and cut-off values see *Material and Methods*). The amount of IFNγ expressing cells is given as percentage of all $CD4^+$ $CD45RO^+$ T cells. Each stimulus used for restimulation is indicated on the x-axis. Individual background values of unstimulated medium controls were already subtracted during data analysis.

For all 11 donors the incubation with the superantigen SEB led to IFNγ production ranging up to 10% of all $CD4^+$ $CD45RO^+$ cells. In all but one case the incubation with PPD also caused detectable IFNγ production which sometimes reached levels comparable to those of the superantigen SEB. After restimulation with ESAT6_CFP10 (1.01) and TB10.4 (1.02) the majority of LTBI showed detectable but low frequencies of IFNγ expression. The median of T-cell frequencies after incubation with the remaining immunodominant antigens were even lower and Hsp65 (1.06) did not induce IFNγ expression in any of the 11 LTBI.

Regarding the DosR regulon only proteins 2.06; 2.10; 2.17 and 2.18 elicited low cytokine responses next to detection limits in one or two donors. The other members of the DosR family (2.xx) as well as the reactivation associated proteins 3.01 / 3.02 did not induce T memory responses above detection limits. The resuscitation related proteins (4.xx) and the reactivation antigen Rv3407 (5.01) led to IFNγ production in some donors but the frequencies of $CD4^+$ $IFN\gamma^+$ memory T cells were also close to unspecific background levels.

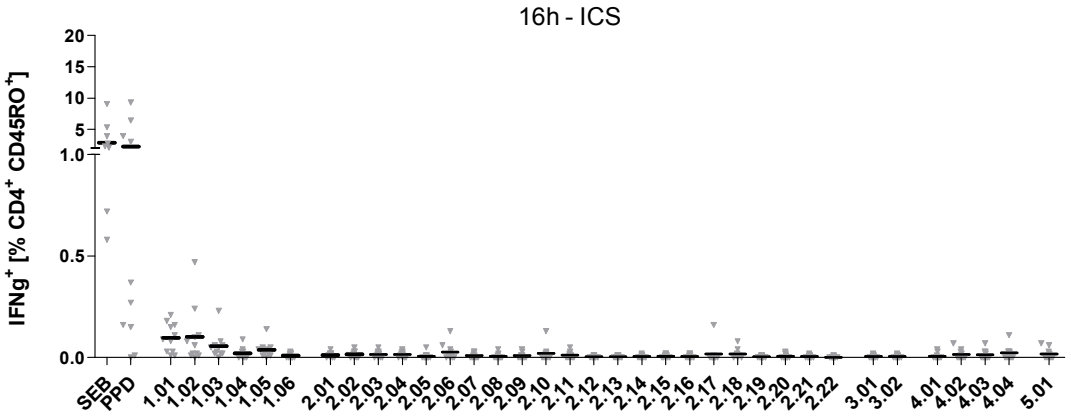


Fig. 12: ICS after 16h *in vitro* incubation of PBMC from LTBI.

$2*10^5$ PBMC from 11 LTBI were restimulated for 16h with 1µg/ml SEB, 10 µg/ml PPD or 5µg/ml *M. tuberculosis* proteins (see fig. 10 for nomenclature). IFNγ expression is depicted for each stimulus as percentage of $CD4^+$ $CD45RO^+$ memory T cells. Individual background values of unstimulated controls were subtracted during data analysis.

There is increasing evidence that short-term assays efficiently detect recent *M. tuberculosis* infection while *in vitro* stimulation for several days is more sensitive for LTBI (Arend, et al., 2007; Cehovin, et al., 2007). Since previous experiments indicated that a 16h restimulation before measurement is optimal for intracellular cytokine detection (Mueller, et al., 2008), 7d of stimulation were combined with a restimulation 16h prior to analysis. For eight LTBI the result of the long-term stimulation followed by ICS were compared to conventional IFNγ ELISA of the supernatant and measurement of proliferation by incorporation of radioactive labeled thymidine (fig. 13). In all approaches the incubation period was 7d and the protein concentration was 10µg/ml for PPD as well as the recombinant *M. tuberculosis* proteins. ICS, ELISA and proliferation assays were able to detect T-cell responses after incubation with classical as well as latency associated antigens (fig. 13a, b, c). Moreover the results of all three methods correlated. IFNγ responses and proliferation were detectable after incubation with PPD and all of the immunodominant antigens in the majority of LTBI. Compared to the short-term ICS assay the frequencies of IFNγ expressing $CD4^+$ $CD45RO^+$ T cells for PPD and immunodominant proteins, e.g. ESAT6_CFP10 or TB10.4, were significantly increased after prolonged stimulation. In contrast to the 16h time point (DosR responses were generally absent) DosR regulon encoded proteins elicited cytokine responses in the majority of LTBI. After 7d of incubation with latency-associated proteins (2.xx) the frequencies of IFNγ

expressing memory T cells even exceeded those of known immunodominant antigens. The reactivation and resuscitation associated proteins (3.xx, .xx, 5.01) showed a similar distribution. Exceptions are 2.01 and 2.15 which did not induce IFNγ production higher than unspecific background. The same pattern was measured by determination of IFNγ in the supernatant and proliferation-assays. The exception is Rv3407 (5.01) leading to discrepancy between ICS and ELISA/proliferation-assays. For the remaining 34 immunodominant and latency-associated proteins the same tendency could be observed by ELISA and proliferation-assays.

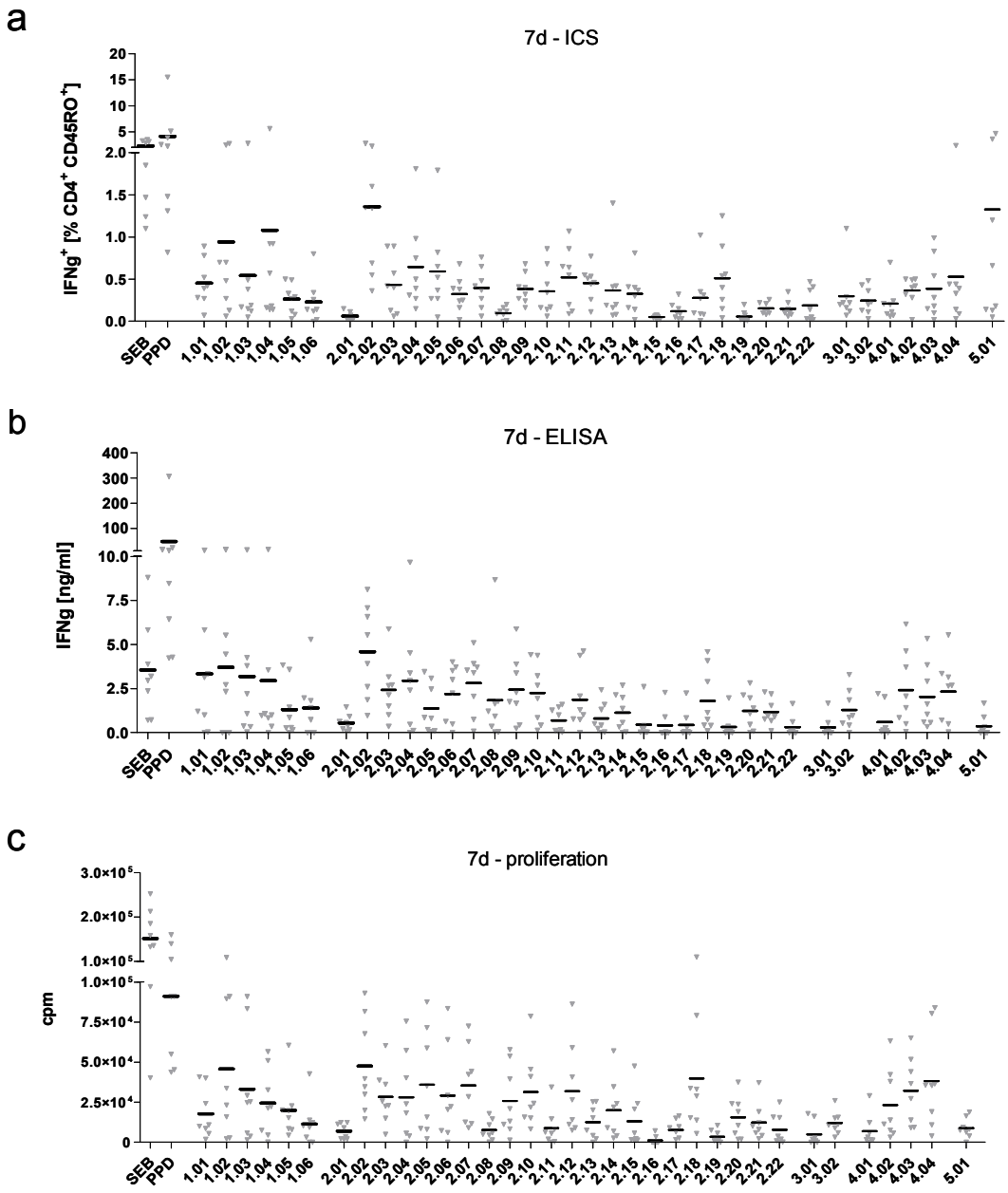


Fig. 13: ICS, IFNγ ELISA and proliferation assay after 7d *in vitro* incubation of PBMC from LTBI revealed amplified T-cell responses.

$2*10^5$ PBMC from eight LTBI were restimulated for 7d with 10µg/ml PPD or 5µg/ml *M. tuberculosis* proteins (see fig. 10 for nomenclature). A second round of restimulation with the respective antigen was performed on day 6 as well as the addition of 1µg/ml SEB which served as a positive control. Individual background values of unstimulated controls were subtracted during data analysis. **a)** ICS after addition of 15µg/ml brefeldin A 12h prior to FACS analysis. IFNγ expression is depicted for each stimulus as percentage of $CD4^+$ $CD45RO^+$ memory T cells. **b)** Commercially available ELISA detecting extracellular IFNγ in the cell culture supernatant after 7d incubation and two rounds of restimulation. IFNγ concentrations are shown for each stimulus. **c)** Measuring proliferation by incorporation of [^{3}H]-labeled thymidine using the 96well Unifilter/TopCount system. Counts per minute (cpm) are depicted for each stimulus.

Since there is the unlikely possibility that prolonged co-culture of PBMC and antigenic proteins could generate "memory-like" T cells *in vitro*, it was essential to ensure that the observed cytokine responses were due to a priming of T cells in the host and no artifact of the long-term stimulation. Therefore the same experiment was performed with non-*M. tuberculosis* infected donors having a negative TST skin test. The results for parallel ICS, ELISA and proliferation are depicted in figure 14. Cellular analysis by ICS revealed IFNγ expressing T-cell frequencies close to detection limits for the eight uninfected controls. Only a single donor showed weak T-cell responses against 2.09, 2.18 and 2.21. In contrast, IFNγ was detected in the supernatant for the majority of LTBI and the majority of *M. tuberculosis* derived proteins. This also held true for the determination of proliferation by measuring the incorporation of radioactive thymidine.

Crucial for the development of a new assay is the signal-to-noise ratio. In our study the signal is represented by the specific T-cell responses towards latency-associated antigens and the noise can be interpreted as the unspecific background for each particular protein after stimulation of PBMC from uninfected donors. All three techniques detected the specific "signals" of PBMC from latently infected donors. Nevertheless ELISA and proliferation assays both detected T-cell activation in uninfected donors as well. Therefore these methods are suboptimal to detect latency-associated T-cell responses after long-term stimulation and ICS turned out to provide the highest sensitivity and specificity.

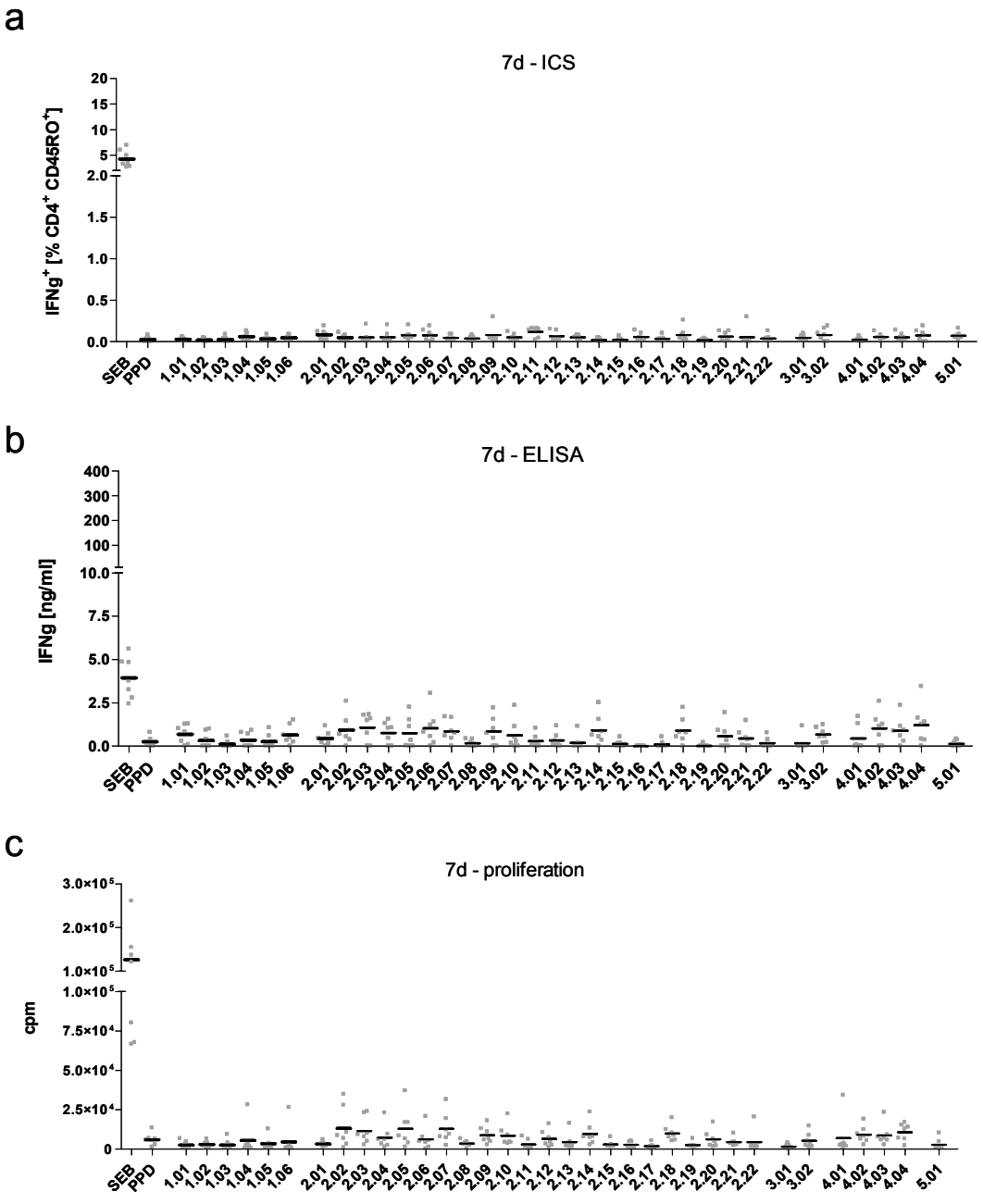


Fig. 14: ICS, IFNγ ELISA and proliferation assay after 7d *in vitro* incubation of PBMC from TST negative donors.

2*10^5 PBMC from eight non-*M. tuberculosis* infected donors (TST neg.) were restimulated for 7d with 10 µg/ml PPD or 5µg/ml *M. tuberculosis* proteins (see fig. 10 for nomenclature). A second round of restimulation with the respective antigen has been performed on day 6 as well as the addition of 1µg/ml SEB. Individual background values of unstimulated controls were subtracted during data analysis. **a)** ICS after addition of 15µg/ml brefeldin A 12h prior to FACS analysis. IFNγ expression is depicted for each stimulus as percentage of $CD4^+$ $CD45RO^+$ memory T cells. **b)** Commercially available ELISA detecting extracellular IFNγ in the cell culture supernatant after 7d incubation and two rounds of restimulation. IFNγ concentrations are shown for each stimulus. **c)** Measuring proliferation by incorporation of [^{3}H]-labeled thymidine using the 96well Unifilter/TopCount system. Counts per minute (cpm) are depicted for each stimulus.

There was the possibility to directly compare T-cell frequencies after 16h and 7d for the same eight LTBI. In order to elucidate the underlying mechanisms the amplified response towards immunodominant antigens after 7d was compared to the response elicited by latency-associated antigens. Basically two explanations may account for differential IFNγ expression between the 16h short-term assay and the novel 7d assay: (i) The prolonged stimulation period followed by a second restimulation boosted weak T-cell responses agianst latency antigens which were below detection limit after 16h; (ii) T cells recognizing subdominant latency antigens in LTBI are at a different maturation stage compared to T cells specific for immunodominant proteins and require a second round of restimulation for optimal activation and cytokine expression.

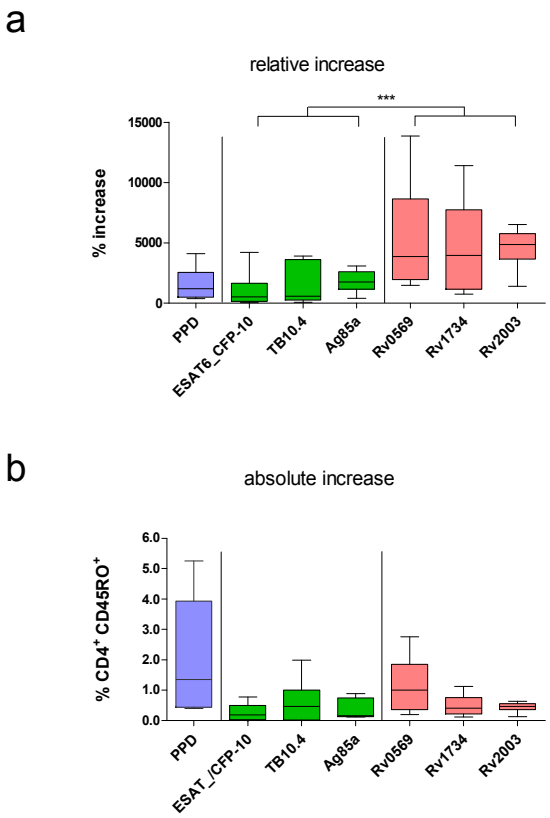


Fig. 15. Absolute and relative increase of IFNγ expressing T cells from 16h to 7d.

Box plots compare relative and absolute increase of IFNγ expressing $CD4^+$ $CD45RO^+$ T-cell proportions. Pair-wise comparisons for six LTBI are shown. The values for PPD are depicted on the left side, three selected immunodominant proteins ESAT6_CFP10, TB10.4, and Ag85a in the middle panel, and three latency-associated proteins Rv0569, Rv1734, and Rv2003 in the right panel. **a)** Individual relative increase (ratio of 7d-response / 16h-response) as percentage of the initial 16 h-response. **b)** Individual absolute increase (difference between 7d- and 16h-responses) of IFNγ expressing $CD4^+$ $CD45RO^+$ memory T cell proportions. *P* value for *** = 0.0002 using the two-sided paired t-test.

As an initial step to address this question the relative and the absolute changes in the proportion of cytokine expressing T cells induced by different stimuli between 16h and 7d were compared in individual donors. Figure 15 indicates the relative increase (ratio of 7d-response / 16h-response) and the absolute increase (difference between 7d- and 16h-responses) of IFNγ expressing $CD4^+$ $CD45RO^+$ memory T-cell proportions. Three immunodominant antigens (left panel) and three latency-associated antigens (right panel) which elicited the highest frequencies of IFNγ expression are shown. The relative increase in terms of percentage of the 16h response was significantly higher in subdominant latency antigens as compared to immunodominant antigens ($p = 0.002$; fig. 15a). On the opposite, the absolute increase in T-cell proportions induced by subdominant latency antigens was comparable to that of immunodominant antigens (Fig. 15b).

6.2.2 Clinical study of selected candidates

The vast majority of the selected antigens are associated with latent stages of *M. tuberculosis* infection. Consequently, the question whether these antigens are differentially recognized by T cells from patients with active pulmonary TB and donors with LTBI arises. For this study the optimized long-term stimulation protocol was used together with the eleven subdominant antigens that induced the strongest responses in LTBI in the former experiments. The characteristics of 22 LTBI, 20 patients with active pulmonary TB and 10 TST negative controls included in this study are given in figure 16.

	LTBI	TB patients	Non-infected donors
Total number	22	20	10
Female	11	10	5
Male	11	10	5
Age range years (median)	28-64 (47)	26-63 (41)	24–55 (36)

Fig. 16: Characteristics of LTBI, TB patients and non-infected donors.

Overview of total numbers, gender and age of donors included to the cross-sectional study. For each of the 52 donors the 7d *in vitro* restimulation assay was performed with a selection of latency-associated proteins of *M. tuberculosis*. For further details about study group criteria see *Material and Methods*.

There is no bias in age or gender among all three study groups. Diagnosis of LTBI is based on a positive TST skin test verified by a positive T-Spot TB. TB patients were HIV negative and blood was drawn either before beginning of drug treatment or before sputum conversion.

Further details and cut-off values are part of the *Material and Methods* section (see *5.1.1 Human subjects*).

The amount of IFNγ expressing $CD4^+$ $CD45RO^+$ T cells after 7d incubation and two rounds of restimulation with the respective antigen are depicted for non-infected controls, LTBI and TB patients (fig. 17.). Individual background values of unstimulated controls were already subtracted during data analysis. All three study groups showed comparable amounts of IFNγ expressing T cells after incubation with the superantigen SEB. The proportion of $IFN\gamma^+$ $CD4^+$ $CD45RO^+$ T cells after 7d of restimulation with PPD reached high levels in LTBI and TB patients comparable to those of SEB. There was no significant difference among patients and LTBI.

$CD4^+$ $CD45RO^+$ T cells from TST negative controls showed no detectable expression of IFNγ after incubation with PPD. The same held true for restimulation with ESAT6_CFP10. For the vast majority of LTBI and TB patients, $CD4^+$ T cell mediated IFNγ responses were detectable but generally lower compared to those induced by PPD. There was no significant difference among both sub-groups and IFNγ producing memory T cells were absent after incubation of PBMC from TST negative donors with ESAT6_CFP10.

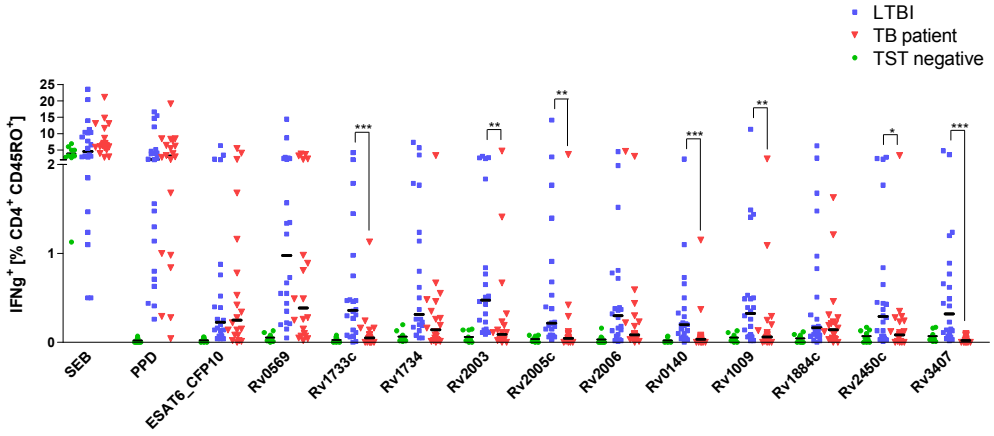


Fig. 17: ICS after 7d *in vitro* incubation of PBMC from LTBI, TB patients and uninfected donors.

$2*10^5$ PBMC from 22 LTBI (blue squares), 20 patients with active pulmonary TB (red triangles) and 10 TST negative donors (green diamonds) were restimulated as already described. Either 5µg/ml of recombinant *M. tuberculosis* proteins, 1µg/ml SEB or 10µg/ml PPD were used. IFNγ expression is depicted for each stimulus as percentage of $CD4^+$ $CD45RO^+$ memory T cells. Individual background values of unstimulated controls were subtracted during data analysis. Nominal two-sided *p*-values for the Mann-Whitney U-test are indicated for the relevant groups (LTBI vs. TB patient) as follows: * $p < 0.03$, ** $p < 0.01$ and *** $p < 0.001$.

Seven out of eleven latency-associated antigens induced significantly higher T-cell responses in LTBI compared to tuberculosis patients ($P < 0.03$). The difference in T-cell recognition among LTBI and TB patients for five of them (Rv1733c, Rv2003, Rv2005c, Rv0140, Rv3407) can be considered highly significant since the particular p-values were below 0.001. Moreover Rv3470 (5.01) exclusively stimulated T-memory cells in LTBI but did not induce detectable IFNγ expressing T cells in diseased patients. The frequency of IFNγ$^+$ memory T cells after incubation of PBMC from TST negative donors with latency-associated antigens was either not detectable or next to unspecific background values.

Figure 18 shows the same data sets for SEB, PPD and the three latency-associated antigens showing highly significant differences among TB patients and LTBI. This time a box-and-whiskers plot is used to illustrate the differences in T-cell mediated recognition for LTBI and TB patients. Arithmetic median, interquartil range as well as overall range are depicted for each data set. Cellular cytokine expression is illustrated as log2 of the percentage of IFNγ$^+$ CD4$^+$ CD45RO$^+$ T cells. By the box-and-whiskers plot the significant higher T cell frequencies for LTBI compared to TB patients for the three latency-associated antigens become obvious. For Rv3407 and Rv0140 there is no overlap in the interquartil range.

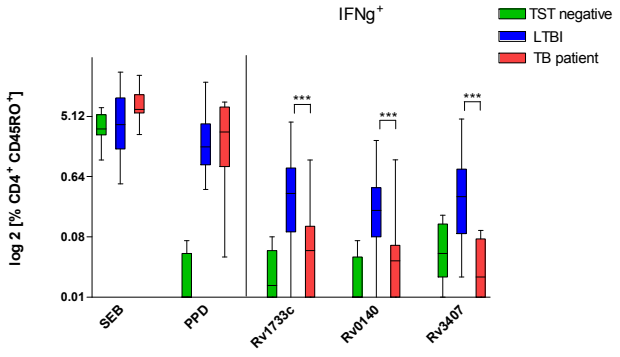


Fig. 18: Significantly higher T-cell responses after 7d incubation with latency-associated antigens in LTBI compared to TB patients.

2*10^5 PBMC from 22 LTBI (blue), 20 patients with active pulmonary TB (red) and 10 TST neg. donors (green) were restimulated as already described. IFNγ expression is depicted for each stimulus as log2 of percentage of CD4$^+$ CD45RO$^+$ memory T cells as box plots. Stimulation with SEB; PPD and latency-associated proteins with highly significant differences in the recognition among LTBI and TB patients (p values below 0.001, fig. 17) are shown. The horizontal line indicates arithmetic median and the box represents the interquartile range of each dataset. The length of the whisker displays overall range (scatter) of each dataset. Individual background values of unstimulated controls were subtracted during data analysis. Nominal two-sided p-values for the Mann-Whitney U-test are indicated for the relevant groups (LTBI vs. TB patient) as follows: * $p < 0.03$, ** $p < 0.01$ and *** $p < 0.001$.

Most striking was the complete absence of recognition of Rv3407 by T -helper cells among all 20 patients with TB whereas approximately half of the healthy LTBI group showed high frequencies of IFNγ producing T-memory cells after restimulation with this protein.

6.2.3 Immunogenic epitopes within Rv3407

In order to identify immunogenic epitopes within Rv3407, synthetic overlapping peptide pools of this protein were generated (for details see *Material and Methods*). A complete list of the synthetic peptides representing Rv3407 and their amino acid sequence is shown in figure 19. The matrix design of the overlapping peptide pools lead to six 'vertical' and five 'horizontal' pools. Each of the 29 peptides of Rv3407 is present in two pools.

a

No.*	Amino acid sequence
P1	MRATVGLVEAIGIRE
P2	TVGLVEAIGIRELRQ
P3	LVEAIGIRELRQHAS
P4	AIGIRELRQHASRYL
P5	IRELRQHASRYLARV
P6	LRQHASRYLARVEAG
P7	HASRYLARVEAGEEL
P8	RYLARVEAGEELGVT
P9	ARVEAGEELGVTNKG
P10	EAGEELGVTNKGRLV
P11	EELGVTNKGRLVARL
P12	GVTNKGRLVARLIPV
P13	NKGRLVARLIPVQAA
P14	RLVARLIPVQAAERS
P15	ARLIPVQAAERSREA
P16	IPVQAAERSREALIE
P17	QAAERSREALIESGV
P18	ERSREALIESGVLIP
P19	REALIESGVLIPARR
P20	LIESGVLIPARRPQN
P21	SGVLIPARRPQNLLD
P22	LIPARRPQNLLDVTA
P23	ARRPQNLLDVTAEPA
P24	PQNLLDVTAEPARGR
P25	LLDVTAEPARGRKRT
P26	VTAEPARGRKRTLSD
P27	EPARGRKRTLSDVLN
P28	RGRKRTLSDVLNEMR
P29	KRTLSDVLNEMRDEQ

* Peptide number according to the position within the primary sequence from C- to N-terminus

b

	Pool 1	Pool 2	Pool 3	Pool 4	Pool 5	Pool 6
Pool 7	P1	P2	P3	P4	P5	P6
Pool 8	P7	P8	P9	P10	P11	P12
Pool 9	P13	P14	P15	P16	P17	P18
Pool 10	P19	P20	P21	P22	P23	P24
Pool 11	P25	P26	P27	P28	P29	

Fig. 19: Design of synthetic peptide pools representing Rv3407.

a) Overlapping 15mer peptides with a shift of 3 amino acids along the primary sequence of Rv3407 have been designed. All 29 peptides represent the whole protein and were synthesized with a purity of > 80% using combinatory chemistry. **b)** After synthesis 11 pools were generated following a matrix design. Hence a single peptide is present in one "vertical" (light grey) and one "horizontal" (dark grey) pool.

In six out of ten LTBI which were preselected for their profound response against Rv3407, a minimum of 0.2 % IFNγ expressing $CD4^+$ T cells was detected after stimulation with at least two of the peptide pools (fig. 20 a – f). In four LTBI (A - D) a major immunogenic epitope could be identified (Fig. 20 a – d) while in two LTBI (E, F) with the weakest response against the Rv3407 protein no prominent responses were observed (Fig. 20 e – f).

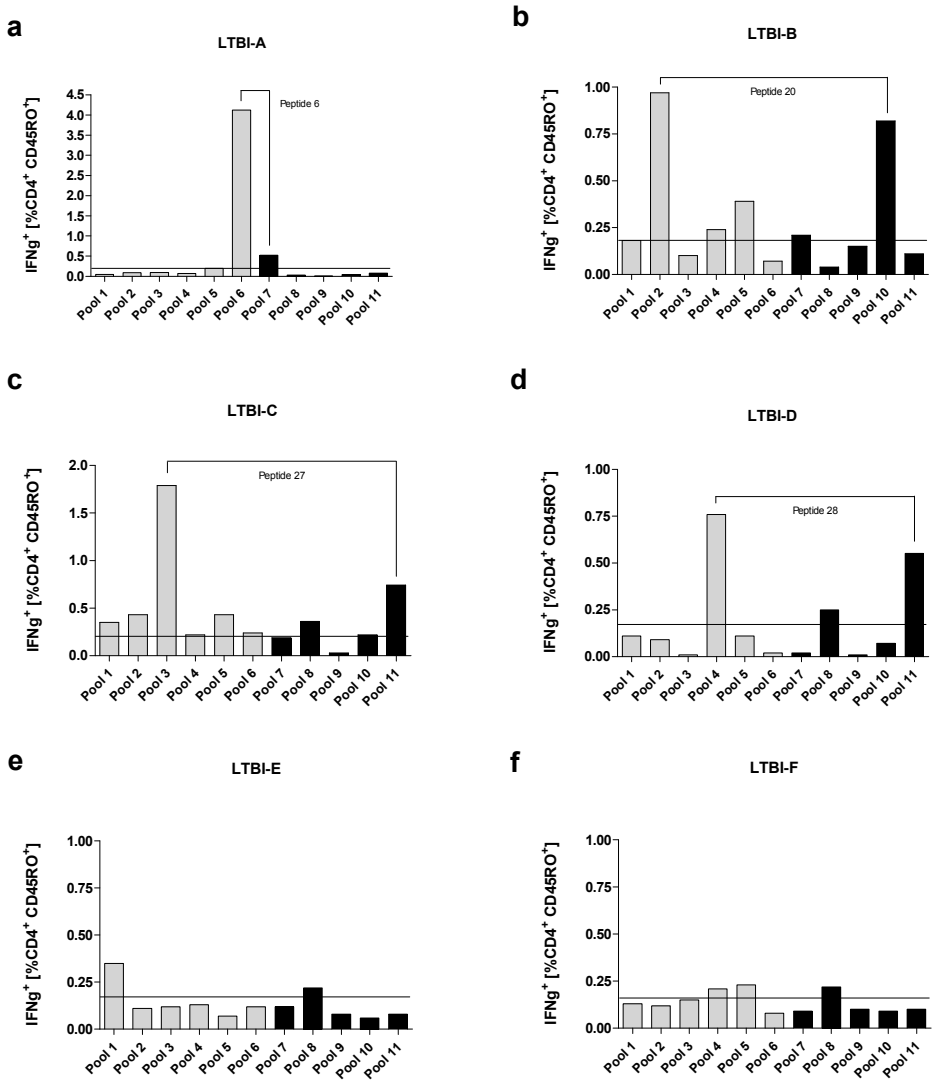


Figure 20. ICS after 7d and two rounds of restimulation with overlapping peptide pools representing Rv3407.

PBMC from six LTBI with Rv3407-specific T cells were restimulated with 15-mer synthetic peptide pools for 7d including two rounds of *in vitro* restimulation. IFNγ expressing $CD4^+$ $CD45RO^+$ T cells measured by FACS are shown after incubation with peptide pools 1 to 6 (grey bars) and pools 7 to 11 (black bars). Each peptide is part of one pool within pools 1 to 6 and of one pool within pools 7 to 11 (see fig.19). Based on the most prominent T-cell responses against the resulting peptides are highlighted for four donors. The horizontal line indicates the arbitrary threshold for positive responses as described in the text. Background values of non-stimulated controls were subtracted for each individual donor.

Notably the peptide 6 identified in LTBI A induced 0.97 % of $CD8^+$ IFNγ-producing T cells as well (data not shown). Aside from this peptide no $CD8^+$ T cells specific for Rv3407 could be identified.

6.2.4 T-cell response under drug treatment

For four patients with active pulmonary TB $CD4^+$ T-memory responses could be measured before beginning of drug treatment (1^{st} time point) and at week six of treatment after sputum conversion (2^{nd} time point). The results are shown for the tested proteins in terms of percentage of IFNγ expressing T cells among all $CD4^+$ $CD45RO^+$ cells (fig. 21). In all patients and at both time points incubation with SEB and PPD induced substantial amounts of IFNγ producing T cells. In most cases detectable frequencies of IFNγ expressing T cells were measured after incubation with ESAT6_CFP10. Only two patients (fig. 21 c + d) had low T-cell frequencies after incubation with ESAT6_CFP10 which were close to detection limits at the 1^{st} time point; for both donors increased T-cell frequencies after stimulation with ESAT6_CFP10 were measured at the later time point. PPD mediated responses were also increased at the end of hospitalization for two patients (fig. 21 a+c), while PPD induced T-cell frequencies remained at the same level for the other two patients (fig. 21 b+d).

Regarding the latency-associated proteins a heterogeneous situation was observed. Generally frequencies of IFNγ expressing $CD4^+$ $CD45RO^+$ T cells after incubation with these antigens were below detection limits before drug treatment and remain low till week six of treatment. Donor b had low but detectable amounts of IFNγ producing T cells before beginning of treatment after incubation with at least three of the eleven proteins (Rv0569, Rv1734, Rv2005c). $IFN\gamma^+$ $CD4^+$ $CD45RO^+$ T-cell frequencies of this patient were increased at the later time point after co-culture with four of the eleven proteins (Rv0569, Rv1734, Rv2003, Rv0140). The strongest increase was observed for Rv0569. Interestingly two out of three proteins eliciting IFNγ expressing T cells at the 1^{st} time point also showed increased T-cell frequencies at the 2^{nd} time point. The remaining patients showed a similar kinetic. Frequencies of IFNγ expressing $CD4^+$ memory cells were below or close to detection limits and increased at the later time point for some of the latency-associated proteins. Such an increase was observed with Rv1734, Rv2003, Rv2005c, Rv1009 for patient c and with

Rv2003, Rv2006, Rv2450c for patient d. Remarkably all three patients possessed increased IFNγ expressing T-cell proportions after incubation with Rv2003 at the 2nd time point.

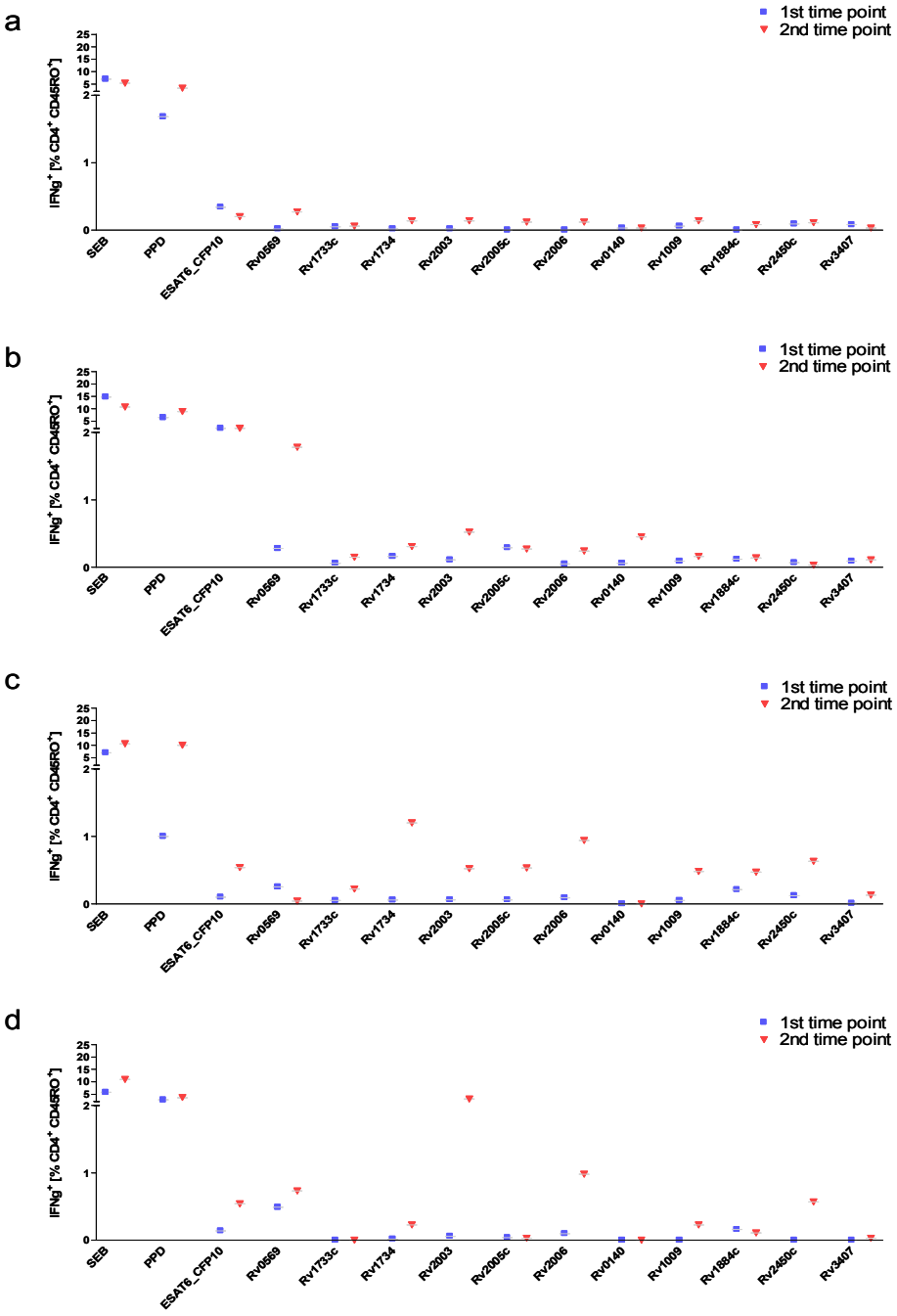


Fig. 21: T-cell response during drug treatment measured by ICS after 7d *in vitro* incubation.

PBMC from four different patients with active pulmonary TB **(a-d)** were restimulated as described. Blood was drawn before/within the first week of drug treatment (time point 1) and at week six of treatment after sputum conversion (time point 2). IFNγ expression is depicted for each stimulus as percentage of $CD4^+$ $CD45RO^+$ memory T cells. Individual background values of unstimulated controls were subtracted during data analysis.

6.2.5 Childhood tuberculosis

Tuberculosis in children is different from the typical course of disease in adults. Since severe disseminated tuberculosis (miliary TB) and TB meningitis are more common among children and small infants, diagnosis of LTBI in these patients goes along with prophylactic treatment with first line antibiotics. This and other dissimilarities regarding the immature immune system lead to an altered "latency" stage different from adults. To investigate the recognition of the latency-associated antigens peripheral blood samples from five healthy children with LTBI and six diseased children with clinically active pulmonary TB were collected. All children were below the age of six.

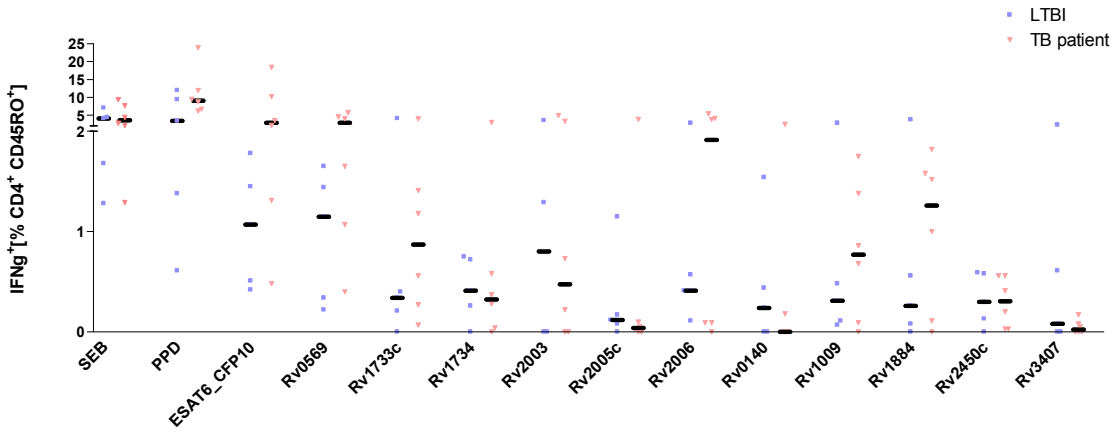


Fig. 22: ICS after 7d *in vitro* incubation of PBMC from children with TB and children with LTBI.

PBMC from six children with active pulmonary TB (age below 6 years) and five children with LTBI (age below 6 years) were restimulated as already described. Either 5µg/ml of recombinant *M. tuberculosis* proteins, 1µg/ml SEB or 10µg/ml PPD were applied. Blood was drawn before/within the first week of treatment. IFNγ expression is depicted for each stimulus as percentage of $CD4^+$ $CD45RO^+$ memory T cells. Individual background values of unstimulated controls were subtracted during data analysis.

For SEB and PPD a similar situation compared to adults could be observed (fig. 22): all LTBI and TB patients showed high frequencies of IFNγ expressing memory T cells and differences among both study groups were absent. Incubation with EAST6_CFP10 did also elicit high amounts of IFNγ producing T cells but the median of $CD4^+$ mediated T-cell responses was higher for TB patients than for LTBI. With regard to the latency-associated antigens IFNγ expressing T cells were detectable for the vast majority of proteins and donors. Five antigens (Rv1734, Rv2005c, Rv0140, Rv2450c, Rv3407) did not induce significantly different frequencies of IFNγ producing memory T cells in LTBI and TB patients.

Moreover the median of T-cell responses measured by IFNγ for the six remaining latency-associated proteins (Rv0569, Rv1733c, Rv2003, Rv2006, Rv1009, Rv1884) was higher in diseased children than healthy LTBI. This is an inverse situation compared to adults where the arithmetic median was lower for TB patients compared to LTBI.

6.3 Characterization of T-cell subsets

The recognition of different *M. tuberculosis* antigens and the absolute frequency of specific T-helper cells are not the only important key factors. A matter of particular interest is the cellular phenotype and other functional properties of antigen-specific T cells involved in eliminating the pathogen. To address this issue single-cell cytokine expression pattern of *M. tuberculosis*-specific $CD4^+$ T cells as well as the stimulatory effect of IL7 were measured in TB patients and LTBI.

6.3.1 Cytokine profiles

The analysis of cellular cytokine secretion patterns became a new focus of immunological research since there is evidence that the amount of poly-cytokine producing T cells may be crucial for protection against viruses and other infectious diseases (Seder, et al., 2008). Our own department recently discovered that the amount of poly-functional T cells in mice vary according to the particular vaccination strategy against tuberculosis and may have an impact on the outcome of infection (unpublished work by C. Desel *et. al.*). Hence cellular cytokine profiles for each particular *M. tuberculosis* Beijing fraction were studied in patients with active pulmonary TB and healthy LTBI. First two prominent T-helper cytokines were analyzed on the cellular level. An extended staining panel allowed the analysis of three different cytokines later.

Simultaneous analysis of two cytokines

In a first step cellular cytokine secretion pattern of $CD3^+$ $CD4^+$ $CD154^+$ cells were investigated for two T_h1 effector cytokines; either IFNγ and TNFα or IFNγ and IL2. For each case figure 23 and figure 24 illustrate data analysis after *in vitro* restimulation with Beijing whole cell lysate or medium alone (unstim.). PBMC from a patient with active TB were incubated for 16h as already described and finally stained for phenotypic markers, CD154 expression and IFNγ/TNFα or IFNγ/IL2. The results are representative for three patients with

active tuberculosis. Measurement of the unstimulated medium control show low unspecific background levels close to detection limits. For all three cytokines a strong cellular expression which correlated with the expression of CD154 was detected. Based on the data sets generated by flow cytometry the cellular cytokine pattern has been calculated and is shown as a pie chart.

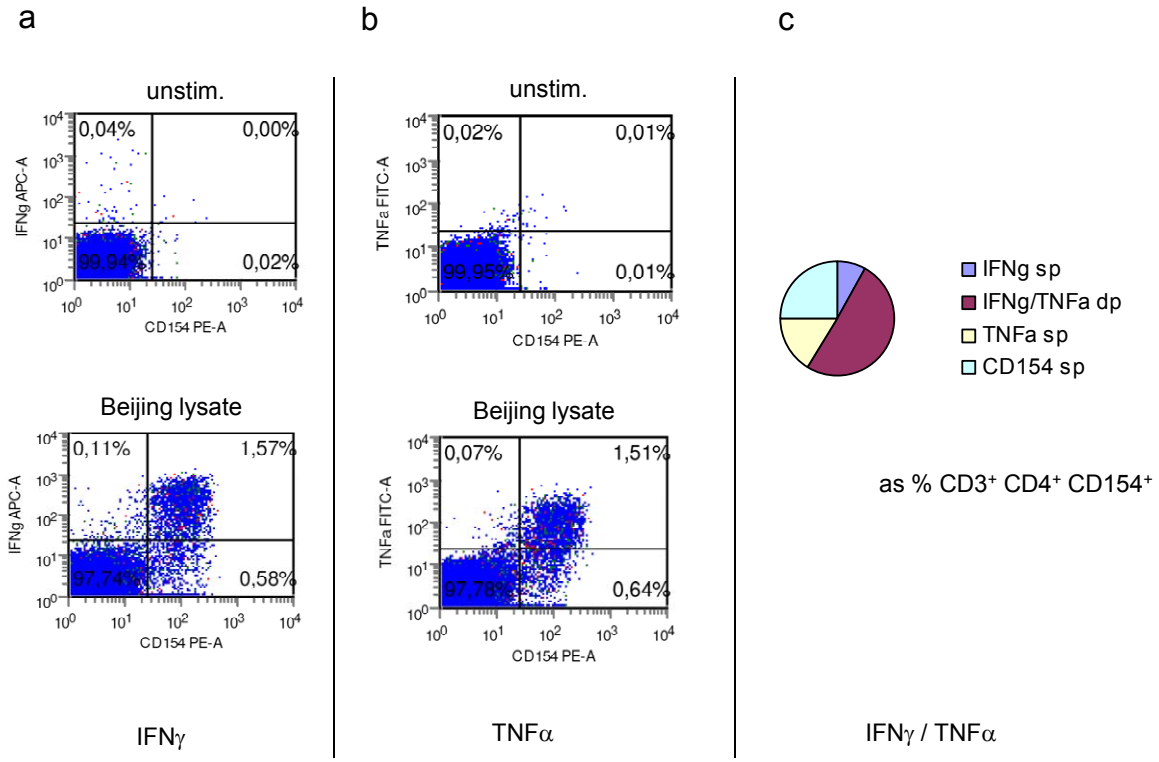


Fig. 23: Cellular T-cell cytokine profiles after 16h of restimulation with Beijing whole cell lysate.

PBMC from a patient with active pulmonary TB were restimulated with Beijing whole cell lysate as already described and analyzed by flow cytometry. Dot plots are gated on $CD3^+$ $CD4^+$ T cells either for unstimulated PBMC or PBMC incubated with Beijing whole cell lysate. **a)** CD154 expression is shown on the x-axis and IFNγ expression on the y-axis. **b)** CD154 expression is shown on the x-axis and TNFα expression on the y-axis. **c)** Calculation of cytokine profiles for $CD3^+$ $CD4^+$ $CD154^+$ T cells. Pie chart represents percentage of $CD4^+$ $CD154^+$ T cells expressing IFNγ (IFNγ sp), TNFα (TNFα sp), both cytokines (IFNγ/TNFα dp) or none of these cytokines (CD154 sp).

The entity of antigen-specific $CD4^+$ $CD154^+$ T cells (activated PBMC) can be divided into three categories: i) cells expressing only one of the cytokines, ii) cells expressing both cytokines simultaneously, iii) cells negative for both of the cytokines expressing only CD154. The simultaneous cellular analysis of IFNγ and TNFα (fig. 23) revealed that the majority of specifically activated T-helper cells ($CD3^+$ $CD4^+$ $CD154^+$) co-express both cytokines after stimulation with *M. tuberculosis* whole cell lysate. Still one fourth of all $CD154^+$ cells do not

express any of these cytokines. The other fourth either express TNFα or IFNγ alone. Notably the amount of TNFα sp cells is approximately two times the amount of IFNγ sp cells.

Analysis of cytokine profiles based on the expression of IFNγ and IL2 (fig. 24) documented a slightly altered distribution. Again the majority of antigen-specific $CD4^+$ T cells simultaneously express both cytokines. The percentage of cells which do not express the cytokines (CD154 sp) is slightly reduced, but the most dramatic differences could be observed for IFNγ sp and IL2 sp cells. Taken together they still reach ~25% of all $CD3^+$ $CD4^+$ $CD154^+$ cells, but the amount of IL2 sp cells is drastically reduced.

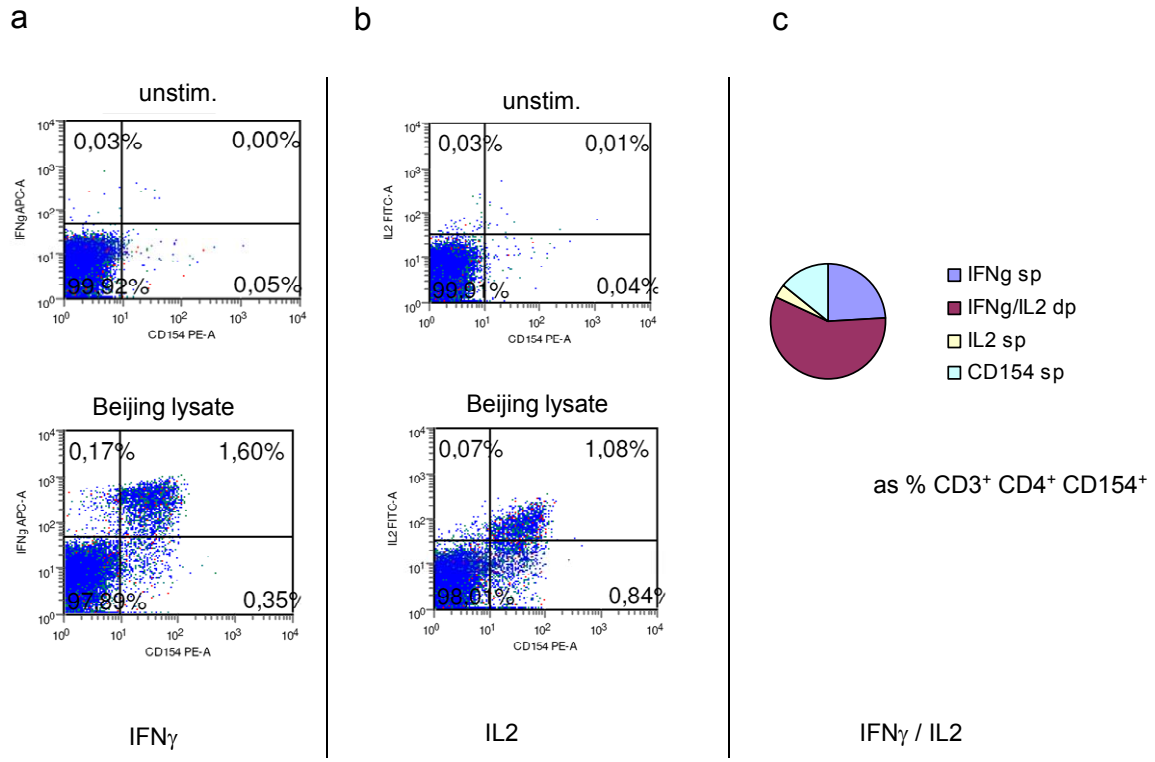


Fig. 24: Cellular T-cell cytokine profiles after 16h of restimulation with Beijing whole cell lysate.

PBMC from a patient with active pulmonary TB were restimulated with Beijing whole cell lysate as already described and analyzed by flow cytometry. Dot plots are gated on $CD3^+$ $CD4^+$ T cells either for unstimulated PBMC or PBMC incubated with Beijing whole cell lysate. **a)** CD154 expression is shown on the x-axis and IFNγ expression on the y-axis. **b)** CD154 expression is shown on the x-axis and IL2 expression on the y-axis. **c)** Calculation of cytokine profiles for $CD3^+$ $CD4^+$ $CD154^+$ T cells. Pie chart represents percentage of $CD4^+$ $CD154^+$ T cells expressing IFNγ (IFNγ sp), IL2 (IL2 sp), both cytokines (IFNγ/IL2 dp) or none of these cytokines (CD154 sp).

In a next step the cytokine secretion pattern after restimulation with the Beijing fractions was analyzed in the same way. For the cellular expression of IFNγ and TNFα the results are shown in figure 25 for three healthy LTBI and four patients with active pulmonary TB.

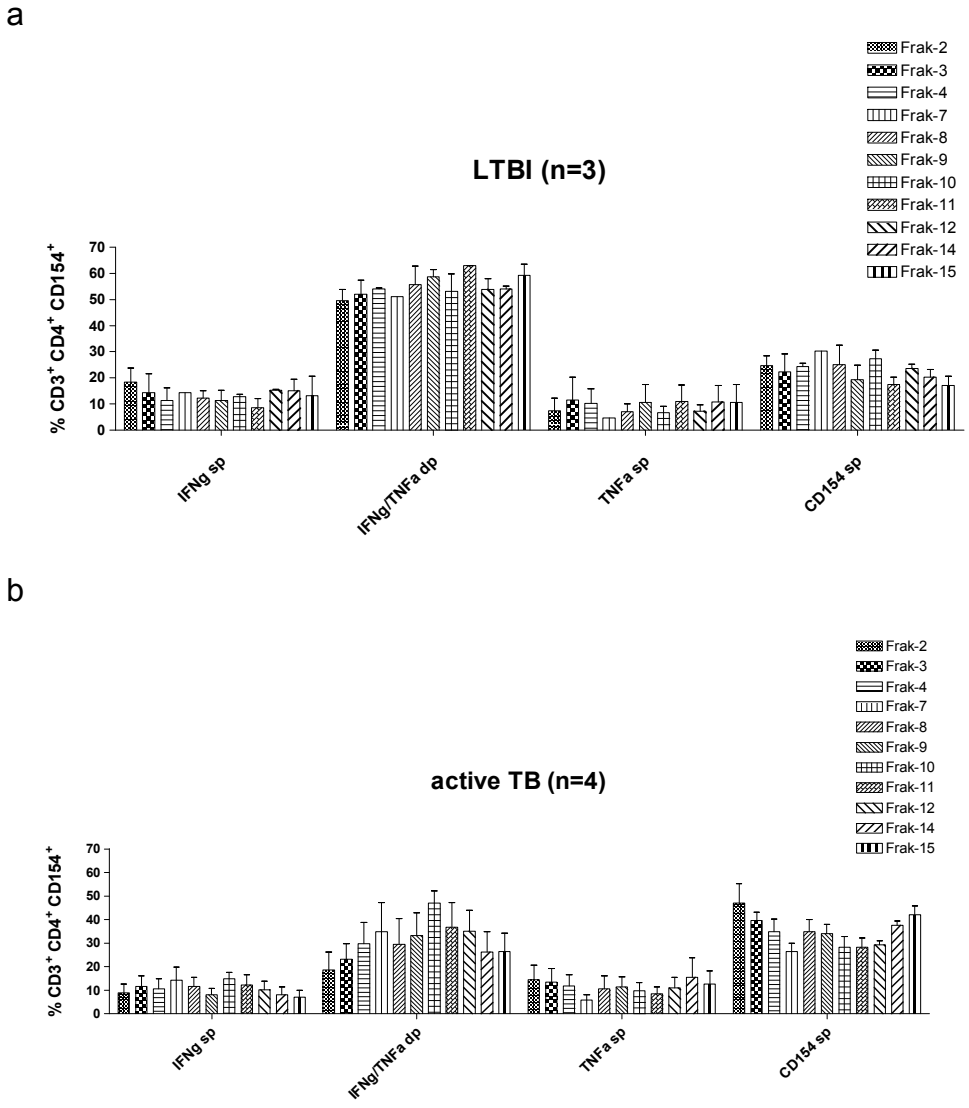


Fig. 25: Cellular T-cell cytokine profiles after 16h of restimulation with Beijing fractions.

PBMC from **a)** four patients with active pulmonary TB and **b)** three LTBI were restimulated with fractionated Beijing whole cell lysate as already described and analyzed by flow cytometry. For each individual fraction the percentage of $CD4^+$ $CD154^+$ T cells expressing IFNγ (IFNγ sp), TNFα (TNFα sp), both cytokines (IFNγ/TNFα dp) or none of these cytokines (CD154 sp) is shown. Bars indicate arithmetic mean and error bars represent standard deviation.

For each fraction the relative amount of IFNγ sp, IFNγ/TNFα dp, TNFα sp and CD154 sp cells is shown as percentage of $CD3^+$ $CD4^+$ $CD154^+$ events within the leukocyte gate. In LTBI the distribution is very similar to former experiments; IFNγ/TNFα co-expressing cells are the majority while CD154 sp approximately represent one fourth. The remaining PBMC are either TNFα sp or IFNγ sp.

For LTBI T-cell responses against different Beijing fractions were similar with regard to their cellular cytokine profile. In contrast, TB patients possessed unique cytokine patterns depending on the particular Beijing fraction. In case of all but one fraction the relative amount of IFNγ/TNFα dp cells was reduced while the amount of CD154 sp cells expressing neither IFNγ nor TNFα was increased. In other words a reduced relative amount of poly-functional $CD4^+$ T cells expressing IFNγ/TNFα in TB patients compared to LTBI was found for the majority of Beijing fractions. This reduction indicates a modified cytokine profile, which was not observed by incubation with Beijing whole cell lysate.

Due to limitations in patients' material the same assay with staining for IFNγ and IL2 could only be performed for a single donor with LTBI and two TB patients. The results are depicted in figure 26 showing the relative amount of IFNγ sp, IFNγ/IL2 dp, IL2 sp and CD154 sp cells among all $CD3^+$ $CD4^+$ $CD154^+$ PBMC. Different to the analysis of IFNγ/TNFα the sub-group of LTBI shows a cytokine profile which differs depending on the Beijing fraction used for restimulation. Some fractions elicited very low poly-functional T-cell responses with IFNγ/IL2 co-expression. Instead the relative amount of CD154 sp cells was increased. Even though the cytokine profiles were different depending on the Beijing fraction, no differences could be observed among LTBI and TB patients. In contrast to the former experiment each individual fraction lead to similar cytokine pattern in LTBI and TB patients.

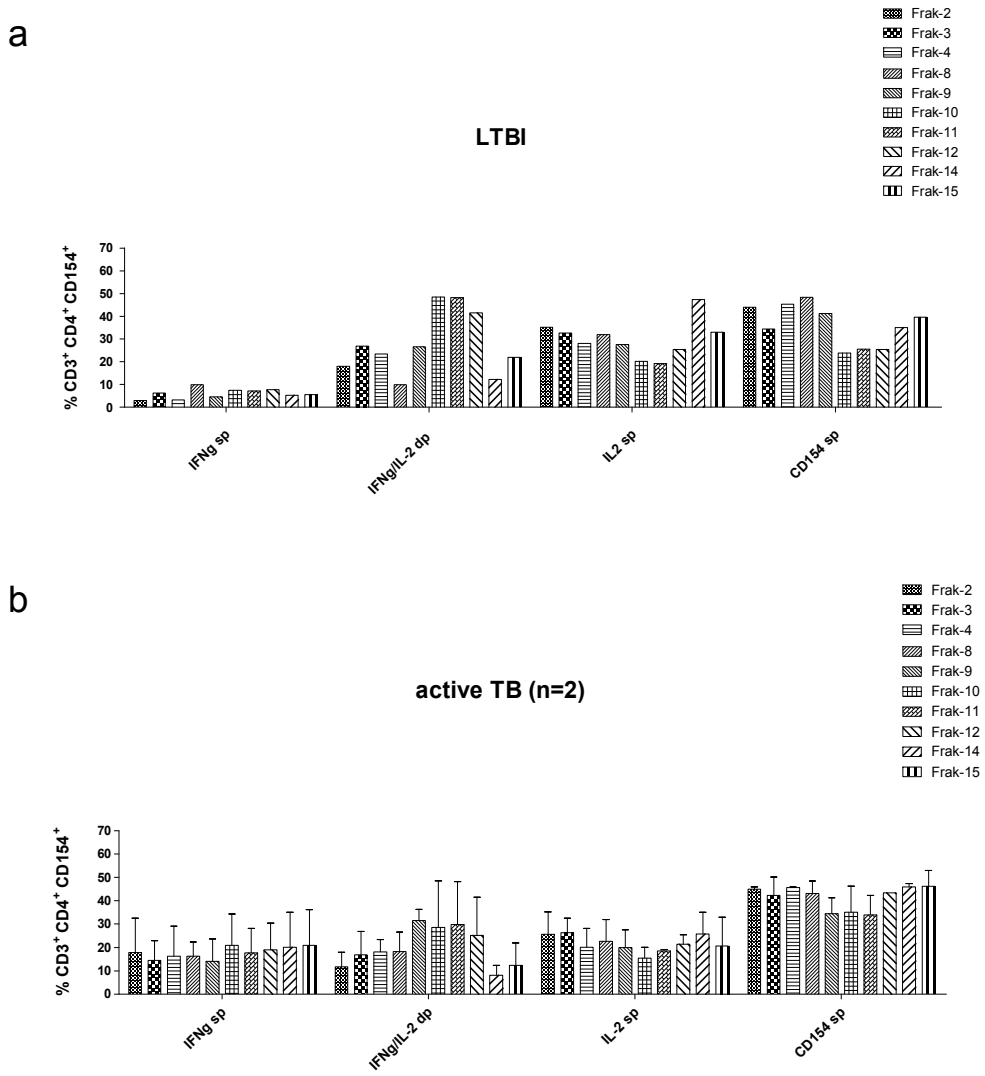


Fig. 26: Cellular T-cell cytokine profiles after 16h of restimulation with Beijing fractions.

PBMC from **a)** one patient with active pulmonary TB and **b)** two LTBI were restimulated with fractionated Beijing whole cell lysate as already described and analyzed by flow cytometry. For each individual fraction the percentage of $CD4^+$ $CD154^+$ T cells expressing IFNγ (IFNγ sp), TNFα (TNFα sp), both cytokines (IFNγ/TNFα dp) or none of these cytokines (CD154 sp) is shown. Bars indicate arithmetic mean and error bars represent standard deviation.

Simultaneous analysis of three cytokines

In order to investigate the cytokine profile for three cytokines simultaneously the combination of staining antibodies was modified. To include a third cytokine the staining for CD154 had to be renounced. This seems unproblematic since former experiments revealed a strong correlation of cytokine expression with CD154 expression. Frequencies of T cells expressing effector cytokines without co-expression of CD154 were either not detectable or close to

detection limits in this assay. Together with the same 16h restimulation procedure a more detailed picture of cellular cytokine pattern after incubation with Beijing fractions could be documented. Figure 27 shows the absolute amount of $CD3^+$ $CD4^+$ T cells expressing IFNγ, TNFα, IL2 or each possible combination of these cytokines using PBMC from a TB patient.

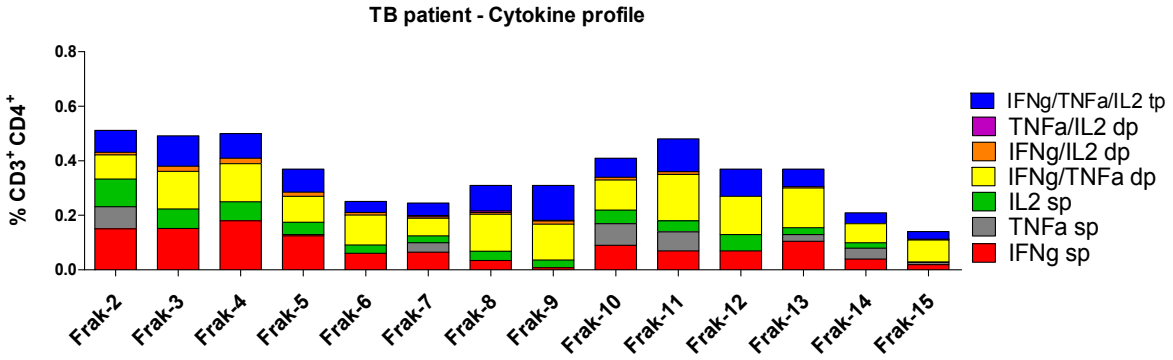


Fig. 27: Cellular T-cell cytokine profiles after 16h of restimulation with Beijing fractions.

PBMC from a patient with active pulmonary TB were restimulated with fractionated Beijing whole cell lysate as already described and analyzed by flow cytometry. For each individual fraction the percentage of $CD3^+$ $CD4^+$ T cells expressing IFNγ (IFNγ sp), TNFα (TNFα sp), IL2 (IL2 sp) or every possible combination of these cytokines is shown. Background values of unstimulated PBMC were subtracted during data analysis.

All 14 fractions elicited cytokine production by PBMC but the height of the $CD4^+$ T-cell response varied from fraction to fraction. This phenomenon was already observed by the more limited analysis of IFNγ expression after *in vitro* restimulation and ICS (fig. 8+9). Differences in the cytokine pattern could be measured, especially with regard to IFNγ/TNFα dp and INFγ sp cells, which is consistent with the former results. Most striking is the high proportion of IFNγ sp cells for fractions 2-4 in combination with a low amount of poly-functional T cells. Each Beijing fraction seems to be characterized by a specific cytokine pattern which is slightly different from the remaining fractions. These differences can also be found between fractions with a comparable over-all CD4 T-cell response (e.g. fraction 2 versus fraction 4).

6.3.2 Effect of interleukin 7

Another observation made in the study of cellular cytokine profiles was the effect of interleukin 7 (IL7) to promote poly-cytokine producing T cells. IL7 is continuously produced and released by human keratinocytes, adherent bone marrow stroma cells as well as thymic cells (Melchers, et al., 1992; Park, et al., 1992). The respective membrane bound 76 kDa receptor (CD127) is mainly expressed on activated T cells, pre B cells and bone marrow

macrophages. An unrelated 90 kDa receptor variant is also expressed on naïve human T cells, but the effect of IL7 on PBMC directly correlates with the expression of CD127 (Armitage, et al., 1992). Signaling through CD127 promotes proliferation and cellular activation, which designates IL7 as a homeostatic growth factor (Guimond, et al., 2005). IL7 enhances the secretion of T_h1 cytokines, namely IFNγ and TNFα, by PBMC and can enhance the *in vitro* generation of cytolytic T lymphocytes (Alderson, et al., 1990; van Roon, et al., 2003). These findings hint to the investigation of cytokine profiles in presence of sub-optimal doses of IL7. Therefore low concentrations of recombinant IL7 (5ng/ml) were chosen since at this concentration IL7 does not lead to proliferation or cytokine production in PBMC without further antigenic stimuli (data not shown). The cellular "three cytokine" profile was determined after 16h co-incubation of PBMC from a TB patient with 10µg/ml Beijing lysate in presence and absence of IL7 (fig. 28). To ensure that IL7 alone had no effect an unstimulated medium control also treated with 5ng/ml IL7 was included. Each individual medium control (+/- IL7) was used to subtract background levels during data analysis for lysate and lysate+IL7.

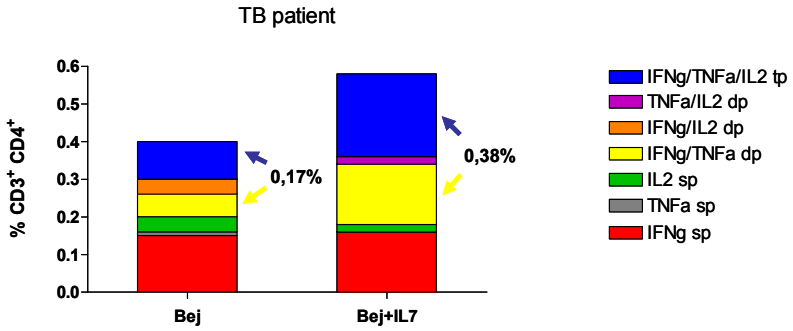


Fig. 28: Modified cytokine profile after 16h of restimulation with Beijing whole cell lysate in presence of IL7.

PBMC from a patient with active pulmonary TB were restimulated with Beijing whole cell lysate as already described and analyzed by flow cytometry. Cells were either incubated with lysate alone or together with 5ng/ml recombinant IL7. The percentage of $CD3^+$ $CD4^+$ T cells expressing IFNγ (IFNγ sp), TNFα (TNFα sp), IL2 (IL2 sp) or every possible combination of these cytokines is shown. Background values were subtracted during data analysis.

Cellular cytokine pattern detected by ICS and FACS analysis are shown in figure 28. Most evident is an increase of the over-all $CD4^+$ T-cell response towards Beijing lysate after low dose IL7 treatment. The percentage of cytokine positive cells rose from ~0.4% $CD3^+$ $CD4^+$ to ~0.55% $CD3^+$ $CD4^+$. In addition to the elevated amount of cytokine positive cells in presence of IL7, the relative cellular distribution (cytokine profile) was also altered. The proportion of

triple positive (tp) T cells expressing IFNγ/TNFα/IL2 (blue) as well as dp T cells producing IFNγ/TNFα (yellow) was increased regarding the absolute amount as well as the relative proportion in relation to the whole $CD4^+$ T-cell response. In this patient sub-optimal doses of IL7 promoted T cells with a poly-cytokine profile leading to an increased over-all memory response against *M. tuberculosis* Beijing lysate *in vitro*.

The identical experiment was done for six patients with active pulmonary TB and the results are illustrated in figure 29. All patients were restimulated with Beijing lysate in presence and absence of IL7. The $CD4^+$ recall response was measured by the expression of INFγ/TNFα/IL2 by ICS and FACS analysis. In all cases an increased percentage of IFNγ/TNFα/IL2 tp and INFγ/TNFα dp cells after treatment with IL7 could be confirmed (fig 29a). The analysis shows the summary of both subsets. While the absolute increase mediated by IL7 can be seen on the left panel, the relative increase is depicted on the right. Depending on the donor the effect was more or less pronounced and caused an increase in IFNγ/TNFα/IL2 triple positive and IFNγ/TNFα dp cells up to +121%.

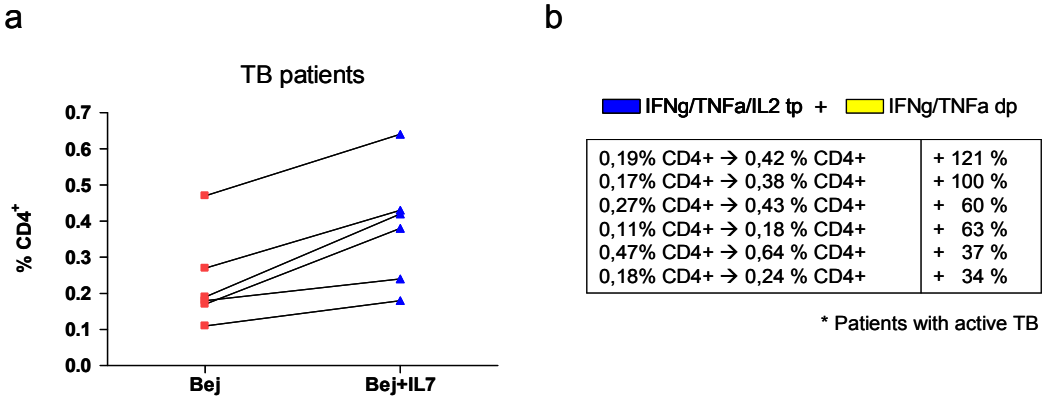


Fig. 29: Analysis of poly-functional T cells after restimulation with Beijing lysate in presence and absence of IL7.

PBMC from six patients with active pulmonary TB were restimulated with Beijing whole cell lysate as already described and analyzed by flow cytometry. Cells were either incubated with lysate alone or together with 5ng/ml recombinant IL7. The sum of T cells expressing IFNγ and TNFα (IFNγ/TNFα dp) or IFNγ, TNFα and IL2 (IFNγ, TNFα, IL2 tp) is shown as percentage of all $CD4^+$ T cells. Background values were subtracted during data analysis.

6.4 Functional *in vitro* assays

Characteristic cytokine profiles as well as the positive effect of IL7 on poly-functional T cells bring a new aspect into play. Their amount represents the quality of the immune response independent from the over-all T-cell frequency. Such qualitative differences could be important for protection of the host against infections (Seder, et al., 2008). But questions regarding the *in vivo* relevance cannot be answered by descriptive clinical studies. It is crucial to understand the impact of T cell sub-populations with different functional properties on the outcome of infection and their role in protecting the host efficiently. Since the typical course of disease and especially the latent stage of infection differ in mice and humans, functional *in vitro* assays are needed. They should allow direct evaluation of positive and adverse effects of different stimuli (e.g. cytokines) or T cell sub-populations (e.g. poly-functional T cells) on the ability of immune cells to kill intracellular mycobacteria.

6.4.1 Methodology

To establish a functional *in vitro* assay human monocyte-derived macrophages infected with mycobacteria were chosen as "target cells". The use of primary cells rather than immortalized human macrophage cell lines allows the generation of autologous *M. tuberculosis*-specific PBMC as "effector cells". An additional feature of this assay is the use of eGFP expressing mycobateria. This allows final read-out of bacterial load by FACS analysis instead of plating bacilli on agar medium followed by determination of colony forming units (CFU) three weeks later. The advantage in time saved by direct FACS analysis is an added bonus but the initial idea was to include phenotypic markers for macrophages and T cells to gain additional information about the survival of macrophages. In combination with bacterial growth analysis this approach aims at discriminating between killing of intracellular mycobacteria with or without macrophage collateral damage. Additionally this approach should allow the analysis of infected macrophages with high and low mycobacterial burden. Figure 30 gives a schematic overview about the assay. It is characterized by two simultaneous processes: i) the generation of monocyte-derived macrophages for infection with eGFP expressing mycobacteria, ii) the long term incubation of autologous PBMC with different specific and unspecific stimuli (i.e. cytokines, *M. tuberculosis* lysate, *M. tuberculosis* derived proteins).

The final assay is carried out by co-incubation of infected macrophages (targets) together with activated autologous PBMC (effectors) at different ratios for 16h-36h. Initially, FACS analysis and determination of CFU was done in parallel as a proof of principle.

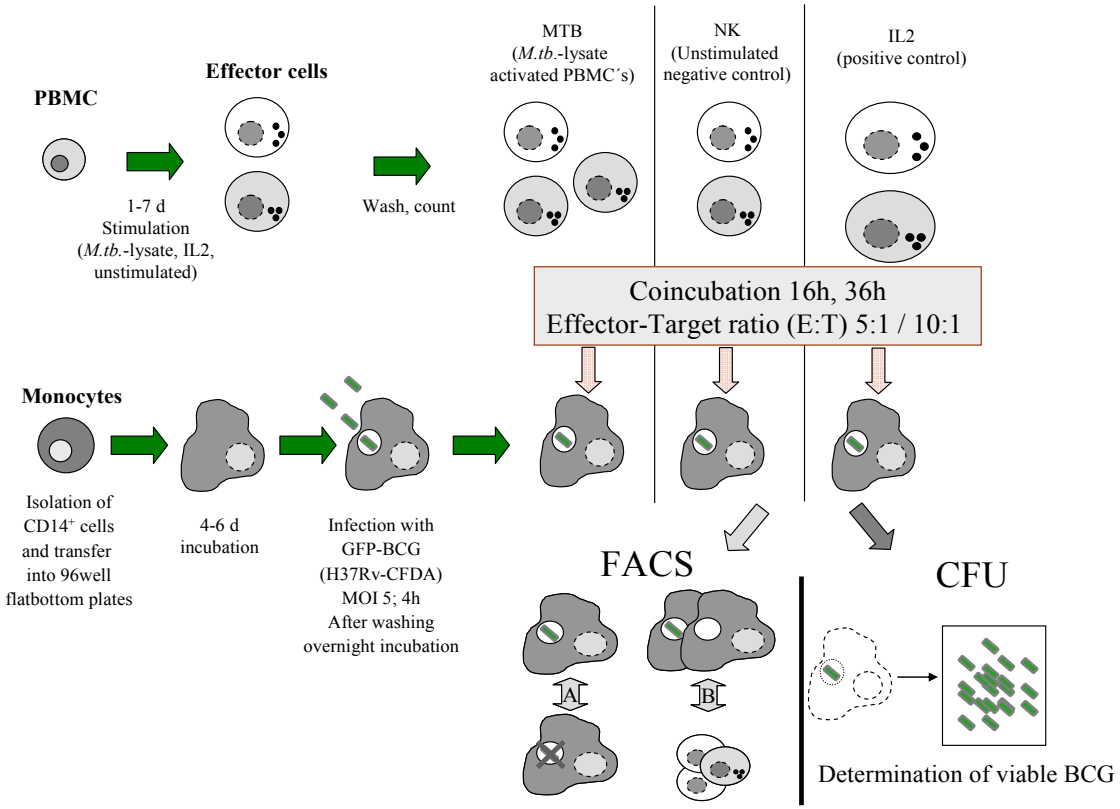


Fig. 30: Schematic overview of the *in vitro* killing assay.

The *in vitro* killing assay is characterized by two parallel steps dealing on the one hand with the generation of monocyte-derived macrophages (upper part) and the stimulation of autologous PBMC to gain effector cells (lower part) on the other hand. After 4-6d the final assay is performed by infecting macrophages with eGFP expressing mycobacteria. The infected macrophages represent targets and were co-incubated for 16h to 36h with different amounts of autologous effector PBMC. To evaluate the ability to kill intracellular mycobacteria, FACS analysis is performed after co-culure, verified by conventional counting of colony forming units (CFU) on plates.

A unique feature of this assay is the analysis of flow cytometric data after co-culture (fig. 31): the staining for phenotypic markers together with the characteristic size (FSC) and granularity (SSC) provide a tool for clear-cut discrimination of PBMC and macrophages. In a first step the proportion of eGFP expressing cells within the macrophage gate is determined. A marker for differentiated macrophages (i.e. CD14 or CD11b) is used in parallel. The amount of $eGFP^+$ macrophages is then compared between unstimulated PMBC and PBMC activated by *M. tuberculosis* lysate or T_h1 cytokines. A reduced percentage of $eGFP^+$ marcophages goes along with a lower bacterial load compared with unstimulated PBMC. This reduction can be

due to specific killing of intracellular mycobacteria with survival of macrophages (conversion from eGFP$^+$ to eGFP$^-$) or caused by the death of infected macrophages. To address this issue effector:target ratios (E:T ratio) were calculated in terms of absolute number of PBMC divided by absolute number of macrophages. Since the number of PBMC remains constant throughout the whole assay, a change in the E:T ratio can only be explained by an altered number of vital macrophages. Consequently an increase in the E:T ratio represents killing of macrophages. The reduction of bacterial burden can be easily verified by determination of CFU and serve as an intrinsic control, but the information about the collateral damage of infected macrophages is a unique feature of this assay. To establish the assay eGFP expressing BCG was used as a surrogate, but in later stages it can be replaced either by carboxyfluorescein diacetate[1] (CFDA) labeled *M. tuberculosis* or recombinant H37Rv expressing eGFP.

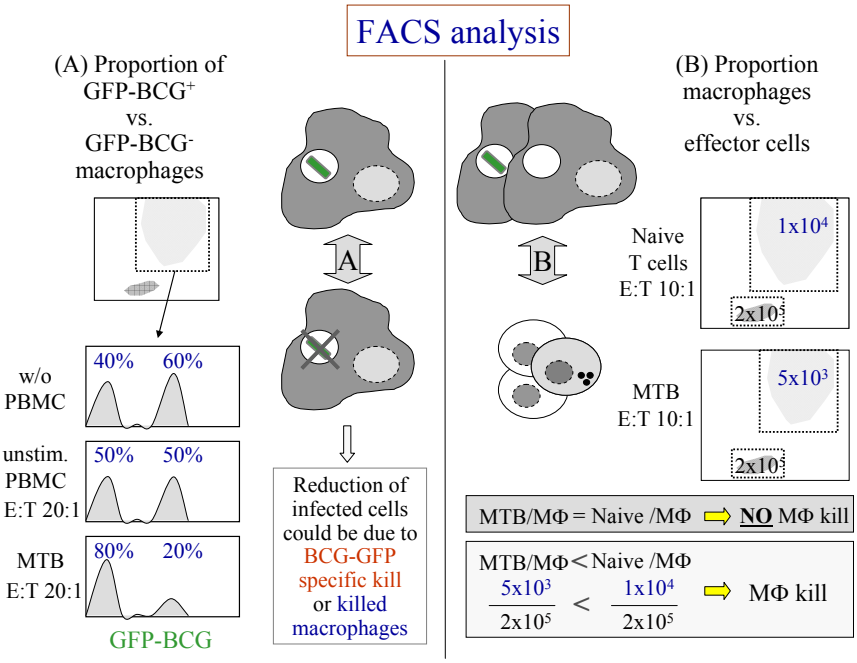


Fig. 31: Schematic overview of data analysis.

Following co-culture, cells were stained for characteristic phenotypic markers in order to identify leukocytes (effector cells) and macrophages (target cells.) Unstimulated PBMC were compared to cells activated by *M. tuberculosis* lysate (MTB) or cells activated by IL2 (lymphokine activated killer cells, LAK). **a)** A reduction of eGFP$^+$ macrophages reflects either specific killing of intracellular mycobacteria or killing of infected macrophages. **b)** Discrimination is possible by calculating the ratio of effector PBMC (constant) and target macrophages (variable). Consequently an increase in the effector:target ratio (E:T ratio) compared to the unstimulated control indicates killing of macrophages.

[1] CFDA is a membrane permeable florescent dye which is cleaved by proteases in the cytosole and stays inside the cell afterwards. It is mainly used for proliferation assays (dilution of the CFDA signal) but due to its stability CFDA can also serve as a cellular tracker.

6.4.2 Functional properties of different T-cell populations

Preliminary experiments should proof that a reduced proportion of eGFP$^+$ macrophages after co-culture with different effector PBMC can be detected by flow cytometry. Therefore monocyte-derived macrophages were infected at a low MOI of 5 for 4h with eGFP-BCG. After infection the target cells were incubated for 16h in presence of unstimulated autologous PBMC or PBMC which had been activated by *M. tuberculosis* lysate and high doses of IL2. The high concentrations of IL2 lead to the generation of lymphokine activated killer cells (LAK) which should reduce bacterial burden by unspecific killing. In all cases 10 times more effector PBMC than macrophages (initial E:T ratio = 10) were applied. These conditions characterized by a low MOI and high concentration of effector cells were chosen to facilitate reduction of bacterial load which is needed to evaluate the feasibility of the new flow cytometry-based killing assay.

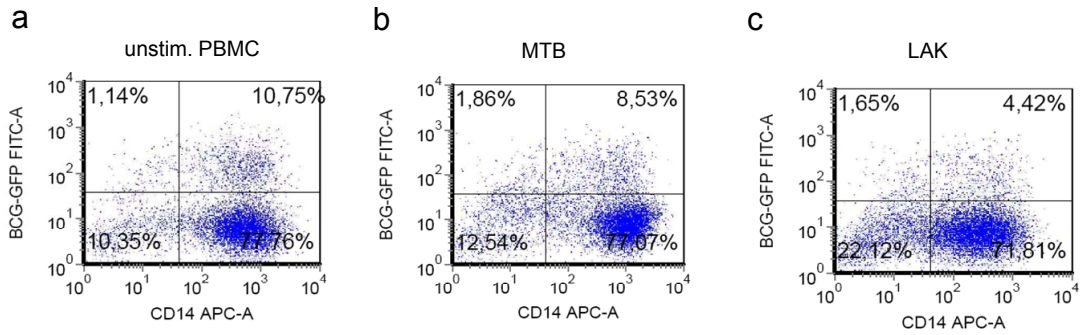


Fig. 32: FACS analysis of macrophages after co-incubation with autologous PBMC.

Monocyte-derived macrophages were infected with eGFP-BCG with a MOI of 5 for 4h. After extensive washing to remove extracellular mycobacteria cells were co-cultured for 16h with autologous PBMC (effector/target ratio: 10:1). **a)** Unstimulated PBMC **b)** PBMC activated by *M. tuberculosis* whole cell lysate (MTB) **c)** IL2 activated killer cells (LAK). The x-axis shows expression of the monocyte/macrophage marker CD14 while eGFP expression is depicted on the y-axis.

FACS analysis of the preliminary experiment is shown in figure 32. Macrophages are gated according to their specific size and granularity in the FSC/SSC. The dot plots indicate CD14 expression on the x-axis and expression of eGFP on the y-axis. The upper right quadrant represents the amount of eGFP$^+$ macrophages as percentage of all gate events. After co-culture with unsimulated PBMC 10.75% of all CD14$^+$ macrophages were also eGFP$^+$. Compared to unstiumlated PBMC the incubation with PBMC activated by *M. tuberculosis* lysate led to decreased proportion of eGFP$^+$ CD14$^+$ macrophages (8.53% eGFP$^+$ macrophages).

The most pronounced reduction was achieved by IL2 activated LAK cells. In this case only 4.42% of all CD14+ macrophages were also eGFP$^+$.

Next a direct comparison of FACS data and CFU was employed to guarantee that the proportion of eGFP$^+$ macrophages correlates with bacterial growth on plates. To reduce assay to assay variation, effector PBMC were replaced by rifampicine or streptomycin in order to reduce bacterial burden. Additionally mycobacteria were used at a higher MOI of 20 to achieve better infection efficiency and the macrophage marker CD14 was replaced by CD11b. This was required since preliminary experiments revealed that CD14 could be down-regulated after prolonged cell culture periods (data not shown). The results are depicted in figure 33 for FACS analysis (fig. 33a) and conventional CFU (fig. 33b).

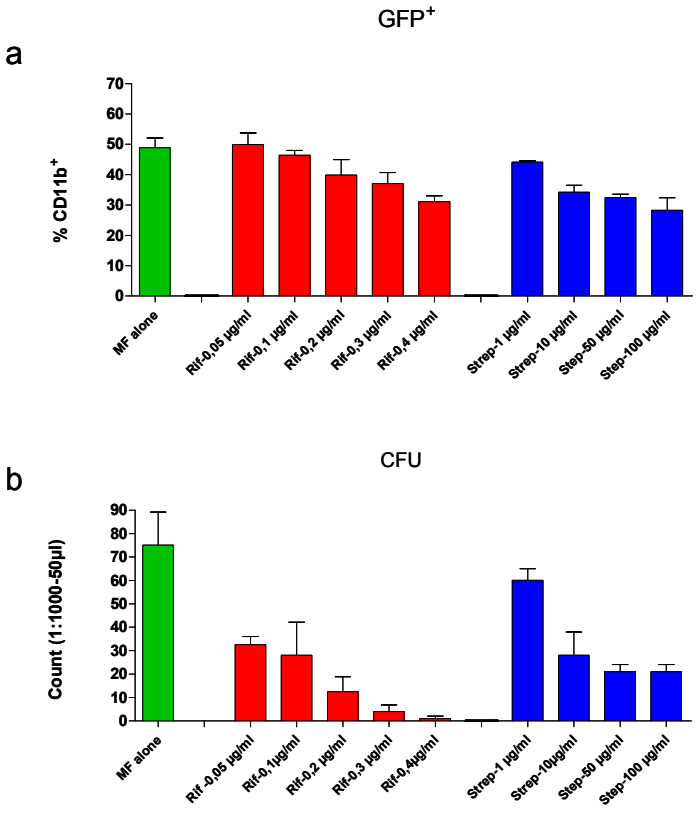


Fig. 33: FACS analysis and determination of CFU after antibiotic treatment.

Monocyte-derived macrophages were infected with eGFP-BCG with a MOI of 20 for 4h and incubated for 16h without antibiotics (green bar), in presence of different concentrations of rifampicine (Rif, red bars) or together with different concentrations of streptomycin (Strep, blue bars). **a)** FACS analysis showing the percentage of eGFP$^+$ events among all CD11b$^+$ events or **b)** determination of CFU on plates. Experiments were done in triplicates, bars represent mean and error is depicted as SD.

For the flow cytometry approach the proportion of $eGFP^+$ events among all $CD11b^+$ events (macrophages) is shown. Macrophages incubated without antibiotics (MF alone) were ~ 48% $eGFP^+$ representing infection efficiency. Treatment with the lowest concentration of rifampicine (0.05µg/ml) did not reduce this amount, but rising concentration subsequently reduced the percentage of $eGFP^+$ macrophages to ~ 30%. In general streptomycin also caused a dose-dependent reduction to levels comparable to rifampicine but at higher absolute concentrations (~ 30% $eGFP^+$ macrophages at 100µg/ml streptomycin). The gradual decrease of bacterial load could be confirmed by CFU. For both antibiotics a similar kinetic depending on the concentration of the drug was observed. Interestingly the number of CFU for untreated macrophages was ~ 73 at a dilution of 1:1000 while lowest doses of rifampicine (0.05µg/ml) were sufficient to reduce the CFU to ~33 at the same dilution. This is in contrast to the FACS analysis which did not document a reduction in $eGFP^+$ macrophages after treatment with 0.05 µg/ml rifampicine.

Since FACS analysis and CFU seem to correlate, PBMC activated by *M. tuberculosis* lysate and the T_h1 cytokine IFNγ were included to the assay (fig. 34). In addition to five different concentrations of rifampicine (0.01µg/ml – 0.5µg/ml) the effect of unstimulated PBMC and lysate-activated PBMC in presence and absence of 50U/ml recombinant IFNγ was also tested. PBMC were all applied at the same initial E:T ratio of 10-1. The broad range of different rifampicine concentrations leads to a typical titration curve based on the FACS data. The lowest concentration has no significant effect on the bacterial load while increasing doses gradually decreased the bacterial burden. The maximal effect was already observed at 0.3µg/ml with a reduction in the proportion of $eGFP^+$ macrophages from ~ 21% to ~ 2%. Similar to the previous experiment determination of CFU confirmed the subsequent reduction of bacterial burden by rifampicine in a dose-dependent manner. In contrast to the FACS data lowest concentrations (0.001µg/ml) were already sufficient to decrease mycobacterial CFU on plates. If target macrophages were incubated in presence of unstimulated autologous PBMC for 16h a slight reduction of $eGFP^+$ macrophages could be shown (from ~21% to ~18%). The most intense reduction was achieved by autologous PBMC stimulated with *M. tuberculosis* lysate (from ~21% to ~13.5%). Hence the priming with lysate has an additive effect to other immune processes also carried out by unstimulated (naïve) PBMC. These general findings could be verified by conventional CFU but it is worth to mention that in a few cases equal

bacterial levels by FACS analysis turned out to be different based on CFU. Most striking is the dissimilarity of FACS analysis and CFU for the correlation between medium control (MF) and macrophages treated with IFNγ (MF+IFN). FACS data did not correlate with the growth of bacteria on plates.

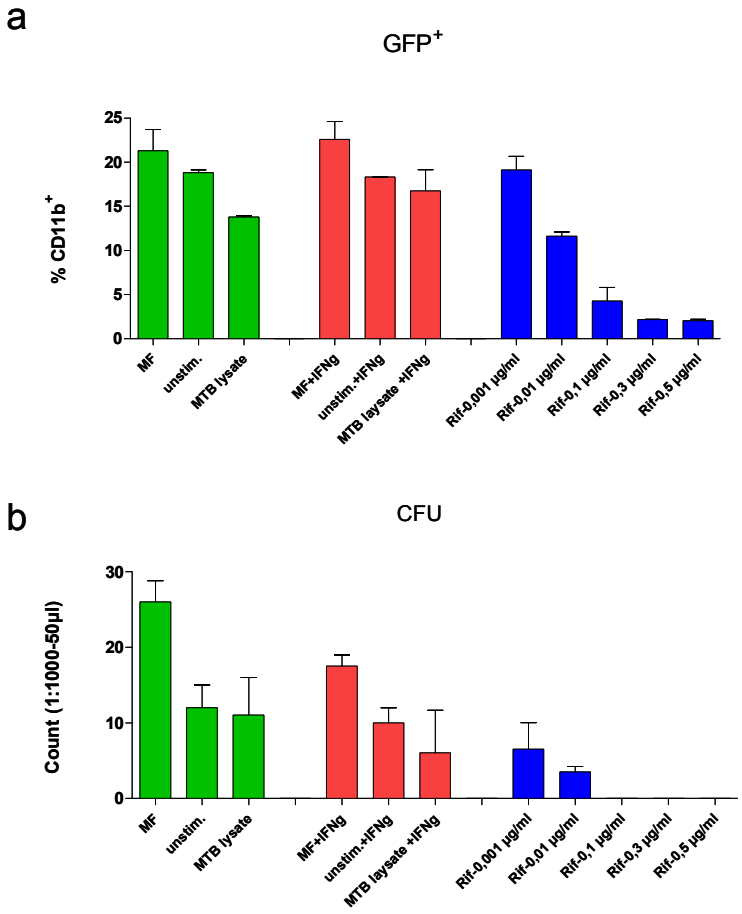


Fig. 34: FACS analysis and determination of CFU after treatment with antibiotics and incubation with autologous PBMC, respectively.

Monocyte-derived macrophages were infected with eGFP-BCG with a MOI of 20 for 4h and incubated with different effector PBMC, antibiotics or cytokines for 16h. Green bars show infected macrophages without treatment (MF) and after co-culture with unstimulated PBMC (unstim.) or PBMC activated by *M. tuberculosis* lysate (MTB). Red bars show the same conditions in presence of 50U/ml recombinant IFNγ. We also included five different concentrations of rifampicine (Rif, blue bars). If autologous PBMC were applied the effector:target ratio was 10:1. Read-out was as either **a)** FACS analysis showing the percentage of $eGFP^+$ events among all $CD11b^+$ events or **b)** determination of CFU on plates. Experiments were done in triplicates, bars represent mean and error is depicted as SD.

In summary, the results support a general correlation of FACS analysis and CFU. Moreover the ideal titration curve regarding different concentrations of antibiotics demonstrates the potential of the assay. A slight killing effect of unstimulated PBMC and a more pronounced

effect of lysate-activated PBMC was observed. Preliminary results also argue for the killing ability of LAK cells measured by FACS. Within the next experiments E:T ratios were calculated to monitor macrophage survival and different amounts of effector PBMC were applied to investigate whether their effect is dose-dependent.

Figure 35 shows flow cytometric data, CFU and E:T ratios of macrophages co-cultured with unstimulated PBMC, IL2 activated LAK cells or PBMC primed with *M. tuberculosis* lysate. Either five times more or ten times more effector PBMC than macrophages were initially applied at the beginning of co-culture period.

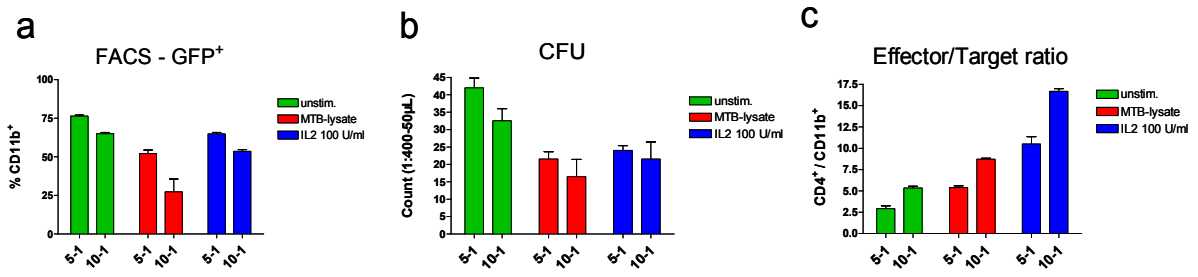


Fig. 35: FACS analysis and determination of CFU after incubation with autologous PBMC.

Monocyte-derived macrophages were infected with eGFP-BCG with a MOI of 20 for 4h and incubated for 16h together with unstimulated autologous PBMC (green bars, unstim.), with PBMC activated by *M. tuberculosis* lysate (red bars, MTB-lysate) or with LAK cells stimulated by 100 U/ml IL2 (blue bars). Autologous PBMC were applied at an initial effector:target ratio of 5:1 and 10:1, respectively. Read-out was as either **a)** FACS analysis measuring $eGFP^+$ events among all $CD11b^+$ macrophages, **b)** determination of CFU on plates or **c)** the final effector:target ratio at the end of co-culture by calculating the ratio of $CD4^+$ events/$CD11b^+$ events based on FACS analysis. Experiments were done in triplicates, bars represent mean and error is depicted as SD.

Unstimulated PBMC (E:T = 5:1) led to ~75% $eGFP^+$ macrophages among the whole population of $CD11b^+$ cells. This proportion is slightly reduced to ~60% by doubling the amount of unstimulated PBMC (E:T = 10:1). In contrast, LAK cells already reduced the amount of $eGFP^+$ macrophages to ~60% at an initial E:T ratio of 5-1, which decreased to ~50% after application of ten times more LAK cells than macrophages (E:T = 10:1). Finally lysate-activated PBMC had the strongest killing effect based on the detection of $eGFP^+$ macrophages. Five times more effector PBMC than macrophages (E:T = 5:1) caused a decrease to ~50% and an E:T ratio of 10-1 led to ~25% $eGFP^+$ macrophages. These results gained by flow cytomertry could be verified by parallel determination of CFU on plates.

In this experiment the E:T ratio was calculated as the quotient of $CD4^+$ events divided by $CD11b^+$ events. For unstimulated PBMC the quotient at the end of co-culture was ~2.5 and ~5

depending on the initial amount of effector PBMC. For the same conditions, IL2 activated LAK cells led to final quotients of ~10 and ~16.5, respectively. Since the amount of $CD4^+$ cells remained constant, the only explanation for an increase in the E:T quotient is massive killing of macrophages. An intermediate quotient of ~5 and ~8 was observed for lysate-activated PBMC, representing slight side damage to macrophages.
It is worth to underline that the novel assay allows discrimination between different killing mechanisms. Lysate-activated PBMC for instance, applied at an E:T ratio of 5:1, reduced the bacterial burden to the same level of LAK cells at an E:T ratio of 10:1. This holds true for FACS analysis as well as CFU. Bacterial growth analysis alone would show a uniform picture, while flow cytometry-based calculation of E:T ratios revealed a side damage of macrophages three times higher for LAK cells compared to lysate-activated PBMC. In other words, flow cytometry provides useful information about qualitative differences in the *in vitro* killing of intracellular mycobacteria.

In order to verify these findings with PBMC from another uninfected (TST negative) donor, a similar experimental setup was used which included LAK cells induced by different concentrations of IL2 (10U/ml, 50U/ml, 100 U/ml). Unstimulated PBMC, LAK cells as well as PBMC activated by *M. tuberculosis* lysate were applied at an initial E:T ratios of 2.5:1; 5:1 and 10:1. Determination of the proportion of $eGFP^+$ macrophages and the calculation of E:T quotients ($CD45^+$ events/$CD11b^+$ events) are given in figure 36. As expected the co-culture together with different amounts of unstimulated autologous PBMC gradually reduced the percentage of $eGFP^+$ macrophages from ~75% to ~52%. The application of lysate-activated PBMC led to a subsequent reduction from ~50% to ~10% in a dose-dependent manner. In contrast, the lowest concentration of IL2 of 10U/ml did not induce LAK cells effectively since the proportions of $eGFP^+$ macrophages were comparable to those of unstimulated PBMC. 50U/ml was sufficient for the induction of LAK cells and especially at high concentrations (E:T ratio of 10:1) these cells were able to reduce the bacterial load, represented as percentage of $eGFP^+$ $CD11b^+$ cells (reduction to ~30%). The highest concentration of 100U/ml IL2 did reduce the amount of $eGFP^+$ macrophages most efficiently. The decrease in bacterial burden was comparable to PBMC activated by *M. tuberculosis* lysate. Regarding the E:T quotients the incubation with lysate-activated PBMC did not cause more damage to macrophages than the incubation with unstimulated PBMC. A higher initial E:T ratio also correlates with a

higher E:T quotient at the end of the assay. Hence information about collateral damage of macrophages was obtained by comparing each particular E:T quotient to the value of unstimulated PBMC.

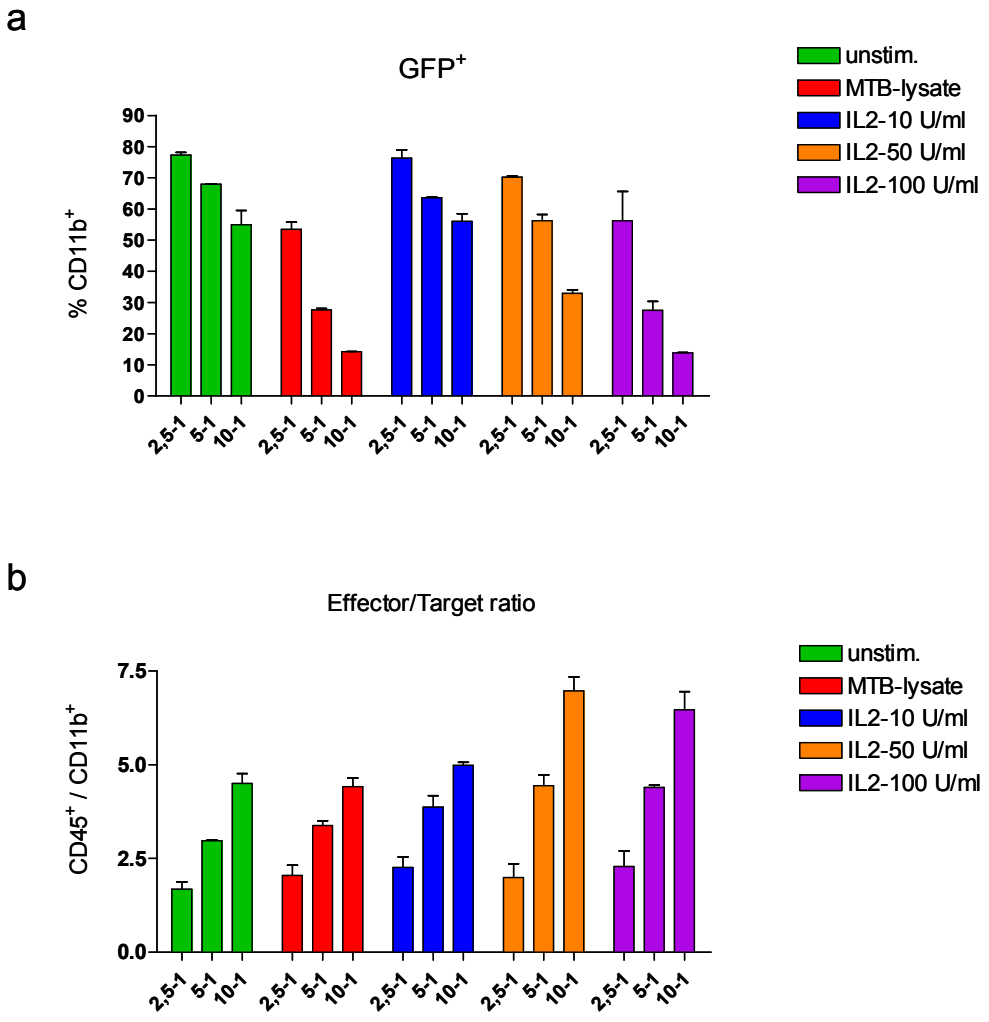


Fig. 36: FACS analysis after incubation with autologous PBMC.

Monocyte-derived macrophages were infected with eGFP-BCG with a MOI of 20 for 4h and incubated for 16h together with unstimulated autologous PBMC (green bars, unsitm.), with PBMC activated by *M. tuberculosis* lysate (red bars, MTB-lysate) or with PBMC stimulated by different amounts of IL2 (blue bars, orange bars, violet bars). Autologous PBMC were applied at an initial effector:target ratio of 2,5:1; 5:1 and 10:1. Read-out was either: **a)** FACS analysis measuring $eGFP^+$ events among all $CD11b^+$ macrophages, **b)** the final effector:target ratio at the end of co-culture by calculating the ratio of $CD45^+$ events/$CD11b^+$ events based on FACS data. Experiments were done in triplicates, bars represent mean and error is depicted as SD

PBMC activated by *M. tuberculosis* lysate or low doses of IL2 (10U/ml) did not cause collateral damage to macrophages (compared to unstimulated PBMC) while LAK cells induced by 50U/ml and 100U/ml of IL2 reduced the absolute number of viable macrophages.

The killing effect on macrophages did positively correlate with the number of effector PBMC applied. Qualitatively this independent experiment verifies the data of the first experiment. PBMC primed with *M. tuberculosis* lysate and high doses of IL2 (50U/ml+100U/ml) gradually reduced the bacterial burden. In case of *M. tuberculosis* lysate the E:Tquotient after co-culture does not argue for massive killing of macrophages. In contrast, PBMC activated by high concentrations of IL2 reduced the amount of viable marcophages, especially if high amounts of effector PBMC were used.

In summary, the *in vitro* killing assay was performed for different donors and with PBMC activated by various stimuli. Figure 37 gives a complete overview of all experiments performed. In some cases the experiments were done with PBMC from the same donor (labeled in green/red). For each single experiment the table indicates whether reduction of intracellular mycobacteria was detectable or not after incubation with antibiotics or PBMC activated by lysate and IL2, respectively. Most important for the feasibility of the new assay is the correlation of FACS data and CFU; the observed reduction in the amount of $eGFP^+$ macrophages should correlate with a reduced bacterial burden determined by bacterial growth on plates. This also includes the correct distribution of bacterial levels within one experiment.

Donor	Killing MTB-lysate	Killing IL2	Killing Antibiotics	FACS/CFU Correlation
086	-	-	Y	Y
086	-	-	Y	Y
083	Y	N	-	-
083	Y	Y	-	Y
083	N	N	-	Y
275	N	N	-	N
276	N	N	-	Y
227	Y	Y	-	N
082	Y	Y	-	Y
008	Y	Y	-	Y
183*	N	N	-	-
570*	N	N	-	N

Fig. 37: Overview of every *in vitro* killing assay performed.

For every *in vitro* killing assay the table shows the identification number of the donor, whether the FACS analysis of GFP^+ macrophages correlated with CFU on plates and finally if a reduction of bacterial load could be observed after i) treatment with antibiotics ii) by LAK cells or iii) by PBMC stimulated with *M. tuberculosis* lysate. Y indicates YES, N represents NO and – stands for "not determined". * = altered protocol / infection with low MOI

Using the final protocol and an infection with a high MOI (upper part of the table) seven of nine killing assays showed correlation of FACS data and CFU. In two cases the bacterial levels gained by flow cytometry could not be verified by CFU. If the distribution of bacterial titers differed only for a single data set the experiment is considered a failure. Remarkably in some cases the generation of effector cells by incubation with IL2 and/or lysate did not reduce the bacterial burden although the same conditions were used. For donor 083 even the same PBMC under the same conditions led to different results.

7 Discussion

Adaptive cellular immune responses are crucial for effective and long lasting protection against the intracellular pathogen *M. tuberculosis*. Consequently the assessment of *M. tuberculosis*-specific T memory responses is a matter of particular interest for vaccination strategies (Reece and Kaufmann, 2008), monitoring of treatment and clinical diagnosis (Lalvani, 2007). Focus of this thesis is the study of human T-cell mediated immune responses against infection with *M. tuberculosis* leading either to active disease or LTBI. By comparing the quantity, quality and specificity of adaptive immune responses among both study groups differences which contribute to the outcome of infection should be identified. Till today suitable markers for the discrimination of LTBI and active disease as well as functional differences responsible for the outcome of infection are still unknown (Jacobsen, et al., 2008). Major findings of this thesis are i) the identification of immunogenic *M. tuberculosis* proteins associated with dormancy, reactivation and resuscitation based on a novel 7 d T-cell assay; ii) comparison between donors with LTBI and TB patients revealed significantly higher T-cell responses against 7 of the 35 tested latency antigens in LTBI; iii) T-cell responses against one of the antigens, namely Rv3407, were exclusively detected in LTBI; iv) MHC genotyping demonstrated an over-representation of HLA-DQ8.1 within the sub-group of LTBI recognizing Rv3407 (*9.2. HLA phenotyping,* p. 123).

7.1 Antigen identification

The first part of this thesis deals with the identification of new *M. tuberculosis* antigens. It can be divided into two independent approaches: fractionated Beijing whole cell lysate and recombinant latency-associated proteins. Relevant in both cases is the comparison of T cell mediated memory responses in latent infection and active TB. Until today various *M. tuberculosis*-derived immunodominant proteins (e.g. ESAT6_CFP10, Ag85a/b and TB10.4) have been identified. Most of these antigens were characterized by restimulation of primed PBMC from infected individuals with culture supernatant, cytosolic lysates or other protein-rich fractions of *M. tuberculosis*. Similar to that, prominent antigenic proteins like ESAT6 and CFP10 were identified in short term culture filtrate by proteomics (Berthet, et al., 1998; Sorensen, et al., 1995).

Since ESAT6 as well as CFP-10 are not present in *M. bovis* BCG, IGRA for the diagnosis of *M. tuberculosis* infection rely on both these antigens (i.e. QuantiFeron GOLD^{TM} and T-Spot TB^{TM}). Even though measurement of IFNγ induced by ESAT6 and CFP10 in LTBI improved the diagnosis, it remained insufficient as a correlate of protection against *M. tuberculosis (Mittrucker, et al., 2007)*. Nevertheless IGRA with prolonged incubation periods were more sensitive to detect past latent TB, discrimination of active TB and LTBI remains impossible by molecular test (Leyten, et al., 2007) Hence, candidate biomarkers which reliably predict protective immunity are urgently needed.

The initial step was to fractionate *M. tuberculosis* Beijing whole cell lysate according to molecular size. The hyper-virulent clinical isolate of *M. tuberculosis* called Beijing was used to mirror the situation in high-endemic areas (Wong, et al., 2007). The potential to elicit antigen-specific immune responses in LTBI and TB patients was then measured by flow cytometry after 16h *in vitro* restimulation for each separate fraction. Qualitative and quantitative differences in antigen recognition among both study groups should be documented.

ICS revealed detectable amounts of IFNγ expressing $CD4^+$ T cells in LTBI after incubation with each of the 11 fractions (fig. 8, p.51). There were only minor differences between the separate fractions. In comparison to LTBI the frequencies of $IFN\gamma^+$ $CD4^+$ T cells were significantly higher in TB patients. This phenomenon can be explained by the different stages of the disease (latency versus active TB) which are possibly accompanied by a different activation status of the immune system. It is worth to speculate that active TB goes along with a higher abundance of *M. tuberculosis* antigens due to replicating bacteria and promote priming of T-helper cells. Nevertheless the relevant reports in the literature are inconsistent. In comparison to LTBI increased levels of IFNγ production after incubation with ESAT6 and CFP10 could be measured in PBMC from TB patients (Arend, et al., 2000; Chee, et al., 2007). In contrast to this, depressed IFNγ levels after restimulation with PPD have been reported by others (Hirsch, et al., 1999). Obviously the particular antigen used for *in vitro* restimulation is crucial for these studies. This might explain why IFNγ responses in the periphery are increased after incubation with certain *M. tuberculosis* proteins while PPD induced responses appear to be static. Additionally the absolute level of IFNγ in cell culture supernatants does not have to correlate with the relative frequency of IFNγ expressing T

cells, since gradual differences in the cellular IFNγ amount can be measured by flow cytometry (data not shown). In summary, T-cell kinetics in humans after exposure to *M. tuberculosis* and the changes during therapy seem to be complex and vary between individuals. For instance, longitudinal studies after point-exposure to TB revealed individual differences in the adaptive T-cell response among untreated donors (Ewer, et al., 2006).

Anyway the intention to document pronounced T-cell responses towards single Beijing fractions was not successful. Although some fractions (i.e. Frak-3; Frak-4; Frak-11) elicited higher frequencies of IFNγ expressing $CD4^+$ T-cells in some TB patients compared to the average of all fractions, these findings were not consistent. Furthermore intermediate recall responses were detected for some fractions (i.e. Frak-8; Frak-9; Frak-10; Frak-12) in some donors which did not allow clear-cut identification of potential candidates.

Despite possible contaminations within the crude bacterial lysate (e.g. TLR ligands or endotoxins) an insufficient separation of the whole cell lysate by gel filtration could be responsible for these observations. The most likely explanation is crude separation of immunogenic proteins by gel filtration since the majority of cytosolic protein yield was recovered within 4 out of 24 fractions (fig 6, p. 48). This would also be an explanation for the observed gradual differences in the strength of the recall response. One could imagine that such a crude isolation in combination with the relatively huge genome of *M. tuberculosis* (4294 genes) does not allow the identification of single antigenic proteins or an effective comparison of *M. tuberculosis*-specific T-cell responses between LTBI and TB patients. In order to achieve a better separation of antigenic proteins within the fractions an ion exchange chromatography was performed following gel filtration. The intention was to further separate the individual fractions according to the charge properties of polar molecules. In preliminary experiments no better separation of the cytosolic protein fraction could be achieved by this strategy (data not shown).

Moreover this study relies on ICS followed by FACS analysis; consequently it includes various effector cytokines but cannot be performed in high-endemic areas where FACS analyzers are still missing. Therefore this approach can be considered as a pilot study and especially the assay conditions have to be adjusted. To evaluate a specific candidate or fraction, detection of IFNγ in the supernatant could be a feasible way which fits the conditions in developing countries.

7.2 Latency-associated antigens

As already mentioned the vast majority of individuals is capable of controlling *M. tuberculosis* infection by specific cellular immunity leading to LTBI. The latent stage of infection is thought to be associated with a dormancy state of the pathogen including the expression of exclusive dormancy related antigens, i.e. DosR encoded proteins (Park, et al., 2003). DosR antigens as well as factors involved in the reactivation and resuscitation of dormant bacilli are candidate biomarkers for LTBI and susceptibility to disease (Biketov, et al., 2000; Chan and Flynn, 2004). To verify the hypothesis that LTBI is also linked to specific T-helper cell responses against such latency-associated proteins, T-cell responses were studied after short-term *in vitro* restimulation of PBMC from LTBI. IFNγ expressing memory T cells were only detectable after incubation with PPD and known immunodominant proteins like ESAT6_CFP10, TB10.4 or Ag85a/b (fig. 12, p. 57). The whole panel of dormancy-, reactivation- and resuscitation-associated proteins (fig. 10, 54) did not induce detectable expression of IFNγ in $CD4^+$ memory T cells after 16h of restimulation (fig. 12, 57). There is increasing evidence that short-term assays efficiently detect recent *M. tuberculosis* infection while *in vitro* stimulation for several days is more sensitive for LTBI (Arend, et al., 2007; Cehovin, et al., 2007; Weir, et al., 1999). Since our own previous experiments indicated that a 16-h restimulation before measurement is optimal for intracellular cytokine detection (Mueller, et al., 2008), 7d of stimulation were combined with restimulation 16h prior to analysis.

Indeed prominent T-cell responses towards the majority of latency-associated proteins as well as amplified T-cell responses towards classical immunodominant proteins were detected by this assay (fig. 13, 59). Consequently the novel 7d assay allows detection of T-memory responses which are missed by 16-h short-term restimulation. Different reasons may contribute to the superiority of the optimized assay. Obviously $CD45RO^+$ memory populations which are present in the periphery at very low frequencies benefit from protracted incubation times, possibly by proliferation leading to detectable T-cell responses after 7d. It is also worth to speculate that a suppressive effect of regulatory T cells could be overcome by these culture conditions. Finally a different cellular phenotype / maturation stage of T cells specific for *M. tuberculosis* latency antigens could be an explanation for the altered restimulation prerequisites of this T-cell subset.

As an initial step to address this question, relative and absolute changes in the proportion of cytokine expressing T cells induced by latency-associated antigens and classical immunodominant proteins were compared between 16h and 7d in individual donors. The relative increase (ratio of T-cell frequencies after 16h and 7d) was significantly higher in latency-associated antigens as compared to classical immunodominant proteins (fig. 15a, p. 62). In contrast, the absolute increase (absolute difference between 16h and 7d) in T-cell proportions induced by latency-associated antigens was comparable to that of immunodominant antigens (fig.15b, p. 62). These results argue against a boost of T-cell responses as the only mechanism involved, since this would also result in absolute differences in comparison to classical antigens. In other words, $CD45RO^{+}$ memory T cells which either recognizes classical immunodominant antigens or latency-associated antigens do not seem to follow the same kinetic. An impressive example is Rv0569 which did not induce detectable IFNγ expression in T cells after 16h while the 7d responses even exceeded those induced by ESAT6_CFP10 (the most immunogenic antigen after 16h restimulation).

In connection with the new restimulation conditions for the detection of T-cell responses towards *M. tuberculosis* latency-associated antigens, the use of ICS, IFNγ ELISA and proliferation assays as readout methods has been evaluated. It turned out that all three assays lead to robust, reliable and comparable results (fig. 13, p. 59), while ICS had by far the highest specificity (lowest 'background' in TST negative uninfected donors; fig. 14, p. 61). In accordance with a previous study (Leyten, et al., 2006) IFNγ was detected in the supernatants of PBMC from LTBI stimulated with latency antigens (fig. 13, p. 59). Notably, and in contrast to ICS, IFNγ was also detected in supernatants of PBMC from some uninfected donors after stimulation with latency antigens (Fig. 14, p. 61).
The second round of restimulation did not cause this higher background in uninfected donors since supernatants collected at day 6 from a subgroup of uninfected donors showed similar IFNγ levels as compared to the day 7 time point after restimulation (data not shown). The possibility that other immune cell populations (i.e. NKT cells, $CD8^{+}$ T cells) were the source of cytokines was excluded by flow cytometry (data not shown). Therefore a possible explanation would be exhaustion or induced killing of these cells (e.g. by apoptosis) due to restimulation 16h prior to analysis (Alsharifi, et al., 2008; Krammer, et al., 2007). Alternatively, these donors may have been false negatives in the T-Spot TB© (although there

is no evidence for this assumption) or these responses may reflect cross-reactivity towards antigens expressed by commensal bacteria (Regner, 2001).

Based on the optimized assay, specific memory responses of $CD4^+$ T cells after incubation with most of the tested latency-associated antigens could be documented in LTBI (fig. 13, p. 59). Hence eleven latency-antigens eliciting highest T-cell responses in LTBI (i.e. Rv0569, Rv1733c, Rv1734, Rv2003, Rv2005c, Rv2006, Rv0140, Rv1009, Rv1884c, Rv2450c and Rv3407) were selected. The amount of IFNγ expressing $CD4^+$ $CD45RO^+$ T cells was compared in patients with active pulmonary TB, LTBI and TST negative donors after 7d of restimulation with these antigens. Seven of the tested antigens (i.e. Rv1733c, Rv2003, Rv2005c, Rv0140, Rv1009, Rv2450c and Rv3407) induced significantly higher amounts of IFNγ expressing memory T cells in LTBI compared to TB patients ($p< 0.03$; fig. 17, p. 64). For Rv1733c, Rv2003, Rv2005c, Rv0140 and Rv3407 these differences were even highly significant ($p< 0.001$; fig. 17, p. 64). People with LTBI in the present study are health care workers at different medical centers in Germany and they can be considered long-term *M. tuberculosis* exposed. All of them remained healthy for years within this environment and therefore their immune response seems to be efficient in containing the pathogen. T-cell responses specifically indicating LTBI argue for their important role in immunity to TB. Of course longitudinal studies regarding the progression of LTBI are needed in addition to this cross-sectional study. Anyway, this is an initial step towards improved detection of "subdominant" antigens in different diseases where reliable biomarkers are still missing and T-cell responses against immunodominant antigens are insufficient for protection.

Different latency-associated antigens elicited T-cell responses in distinct LTBI. Therefore we set an arbitrary threshold of positive T-cell response (0.20 %, i.e. 10-fold higher than the assumed flow cytometric detection threshold of 0.02 %) and determined the minimal cluster of latency-associated antigens which induced a positive response in a maximal proportion of LTBI. Rv1733c and Rv3407 together induced T cell responses in 71.4% of tested LTBI. In contrast, only one of 20 TB patients responded to these proteins (fig 38).

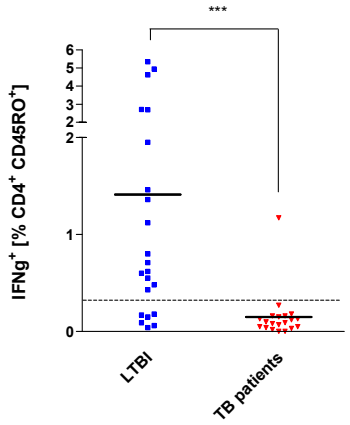


Figure 38. IFNγ expressing T cells after 7d restimulation with Rv1733c and Rv3407.

The sum of the T-cell response elicited by both latency-associated antigens is shown for LTBI and TB patients as percentage of IFNγ$^+$ CD4$^+$ CD45RO$^+$ T cells. PBMC were analysed by ICS after 7d and two rounds of restimulation with either Rv1733c or R3407. The dotted line indicates an arbitrary cut-off of 0.3%.

Discrimination analysis

To determine whether T-cell responses against latency-associated *M. tuberculosis* antigens are sufficient for classification of TB patients and LTBI, we included eleven latency antigens (i.e. Rv0569, Rv1733c, Rv1734, Rv2003, Rv2005c, Rv2006, Rv0140, Rv1009, Rv1884c, Rv2450c and Rv3407) together with ESAT6_CFP-10, PPD and SEB in a discrimination approach. Random forest analysis together with leave-1-out cross-validations across all possible combinations were applied to determine the prediction accuracy and relative importance of each factor for classification (fig. 39a). This analysis revealed a cross-validated prediction accuracy of 83% between TB patients and LTBI. The resuscitation-associated antigen Rv3407 was by far the most influential factor for classification (fig. 39b) (Mollenkopf, et al., 2004)

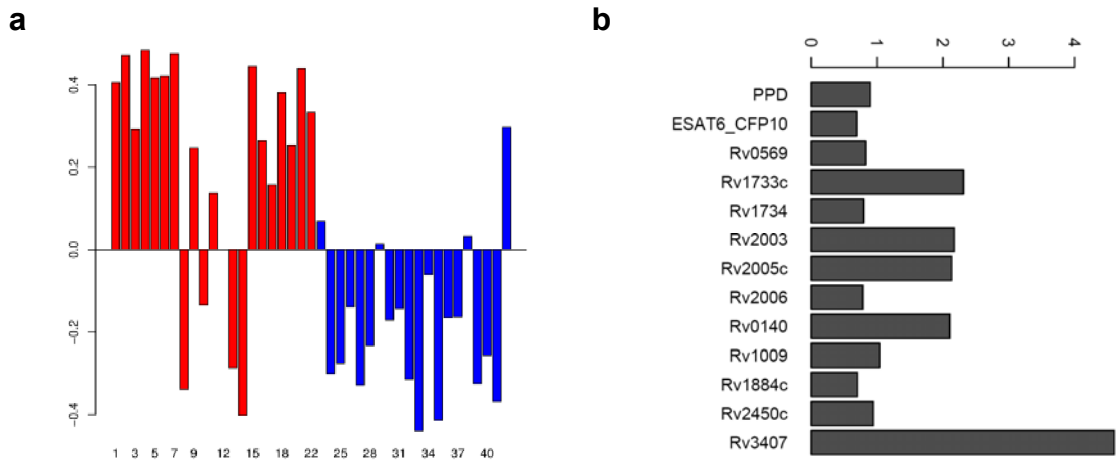


Figure 39. Random forest analysis together with leave-1-out crossvalidations.

Discrimination approach of TB patients and LTBI based on 11 subdominant antigens (i.e. Rv0569, Rv1733c, Rv1734, Rv2003, Rv2005c, Rv2006, Rv0140, Rv1009, Rv1884c, Rv2450c and Rv3407) together with ESAT-6_CFP10, PPD and SEB. **a)** Discrimination approach of TB patients and LTBI. Each bar represents an individual donor. TB patients are shown on the left (red bars), LTBI on the right side (blue bars). The vertical axis indicates the prediction threshold calculated by the random forest analysis. Below the threshold a TB-case is predicted, above the threshold an LTBI-case. The prediction probability is represented as the bar height. **(b)** Mean decrease of class impurity over all trees measured as Gini index indicates the relative importance of each factor for classification.

Based on the cross-sectional study, Rv3407 was the only latency-associated antigen which is recognized in LTBI, but in none of the TB cases. Consequently Rv3407 is the most important factor for discrimination of LTBI and TB patients (fig. 39). To characterize the distinct epitope within Rv3407 overlapping peptide pools were designed. The identification of four different immunogenic peptides by the synthetic matrix pools (fig. 20, p. 67) render the existence of a single 'super-epitope' recognized by the majority of LTBI unlikely. In this context it is worth to mention that in most cases more than a single 'vertical' and a single 'horizontal' pool elicited positive T-cell responses as defined by the arbitrary threshold of 0.2% IFNγ^+ CD4$^+$ CD45RO$^+$ T cells. To approach this issue the pool which led to the highest frequency of IFNγ expressing T cells was used for the identification of the immunogenic peptide. Of course other combinations are possible for some of the LTBI leading to different distinct peptides. Additionally the 7d/two rounds restimulation assay which has been designed for protein antigens seems to be suboptimal for stimulation with peptides. Only six out of ten LTBI which had been preselected for their response to Rv3407 protein also had measurable responses against the overlapping peptide pools. Moreover, only the minority of tested LTBI possessed prominent CD4$^+$ memory responses which

allowed the identification of a single epitope. Possible explanations for the observed dissimilarities between proteins and peptides are i) different pathways of antigen processing and presentation; ii) differences in antigen availability; iii) an altered level of T-cell activation by proteins and peptides. Large particular antigens are taken up and processed by APC leading to TAP-dependent presentation of the containing epitopes to T cells (see *3.2 Adaptive immune responses*). This pathway follows its own dynamic and can be influenced on different levels by signal molecules, e.g. cytokines. The surface expression of MHC molecules as well as molecules involved in antigen processing can be regulated. In contrast, small peptides are loaded directly to MHC molecules on the cell surface without participating in the classical antigen processing pathway. They compete exogenously with other peptides for MHC binding and can replace peptides with low binding affinity (Ackerman and Cresswell, 2004; Hosken, et al., 1989). Consequently the presentation of high affinity peptides follows another kinetic and cannot be regulated directly by the APC.
Intertwined with these differences in processing and presentation is the different amount of available antigen. Intermediate protein concentrations used for restimulation (5 – 10µg/ml) lead to a limited availability of potential epitopes while high concentrations of 15-mer synthetic peptides (1 – 5µg/ml) needed for *in vitro* restimulation provide high amounts of potential T-cell epitopes. All these dissimilarities could result in differences regarding the signal strength sensed by the TCR.
One could speculate that long-term stimulation together with a second round of restimulation lead to activation induced cell death or T-cell anergy for peptides which is not the case for proteins.

Despite the suboptimal assay conditions it could be shown that distinct peptide epitopes in different LTBI were recognized by antigen-specific T cells. Ongoing studies will determine whether this is due to differences in the HLA-phenotype. At this stage high resolution MHC genotyping by polymerase-chain-reaction (PCR) was performed for nine donors with LTBI which recognize Rv3407 protein (*9.2. HLA phenotyping*, p.123). These preliminary results indicate that there are no significant differences in the distribution of MHC class I haplotype from the reference group (German/European population). For MHC class II an elevated amount of donors carrying the HLA-DQA1*0301/DQB1*0302 allele was detected [78% compared to 9.9% for the average of the German population (Kimiyoshi, 1991)]. Regardless

of the distinct epitope recognized within Rv3407 the fact that HLA-DQ8.1 (synonym for the combination of HLA-DQA1*0301/DQB1*0302) is overrepresented among a subgroup of LTBI give rise to new questions and speculations. Especially because memory responses towards Rv3407 mediated by $CD4^+$ T cells seem to correlate with latent infection and possibly with long-term protection against active pulmonary TB.

Genetic factors determining susceptibility or resistance to *M. tuberculosis* infections have been studied extensively. Recent findings indicate that susceptibility to mycobacterial infections is caused by mutations affecting the function of the IFNγ as well as the IL12 receptor (Levin and Newport, 1999). Moreover susceptibility to TB has been linked to a loss-of-function polymorphism in the human P2X7 gene which impairs the ability of macrophages to kill intracellular *M. tuberculosis* (Britton, et al., 2007). Activation of the P2X7 receptor, an ATP-gated Ca^{2+} channel, leads to the formation of pores, the activation of phospholipase D and the induction of apoptosis with death of the infecting mycobacteria.

Molecular defects affecting the regulatory subunit of the NFκB inhibitor (IκB) kinase complex also predispose to server mycobacterial infection (Orange and Geha, 2003). This results in an impaired NFκB activation and reduced responsiveness to TLR activation and TNFα.

Genes encoding the vitamin D_3 receptor, natural resistance-associated macrophage protein (NRAMP1) and mannose binding lectin (MBL) have been associated with TB susceptibility in multiple studies (Fernando and Britton, 2006). All these polymorphisms can be linked to functional deficiencies of the host immune system. Vitamin D_3 is an immunomodulatory hormone that can activate human monocytes and suppresses the growth of mycobacteria (Rook, et al., 1986; Tsoukas, et al., 1984). NRAMP1 plays an important role in macrophages responses to intracellular infections, including the increased production of RNI and ROI, the regulation of MHC II molecules as well as the production of pro-inflammatory cytokines (Blackwell, et al., 2000). Finally MBL is a complement-activating protein and opsonizing agent (Janeway, 2005). Single studies also reported an association of susceptibility to TB with TLR-2 and IL1Rα mutations as well as TAP2 polymorphisms (Bellamy, et al., 1998; Ogus, et al., 2004; Rajalingam, et al., 1997).

Despite genetic predisposition factors which affect basic immunological functions some links to the HLA genotype have been reported so far. HLA-C, the main ligands for killer inhibitory receptors (KIR), are divided in two allotypes. The group 1 allotypes were found to

be more prevalent among patients with TB, which suggests a possible inhibition of NK cell activity against the infected target cells (Bothamley, 1999). In a case-control study of an Indian population, susceptibility to TB was found to be associated with HLA-DR2 (Bothamley, et al., 1989; Rajalingam, et al., 1996). Additionally a Cambodian study showed a significant association of HLA-DQB*0503 allele and susceptibility to TB (Goldfeld, et al., 1998). Recently a new X-linked recessive form of Mendelian susceptibility to mycobacterial disease has been reported (Bustamante, et al., 2007).
A link to HLA-DQ8.1 has not been found so far. Since this analysis focuses on a subgroup of LTBI which possibly possess an advantageous phenotype of adaptive immunity, the overrepresentation of DQ8.1 could be missed considering the entire group of LTBI. This issue has to be addressed in large-cohort studies to receive more reliable data.

For some of the patients with active pulmonary TB the T-cell response against latency-associated *M. tuberculosis* proteins could be compared at the beginning of treatment and six weeks later after sputum conversion. This comparison revealed two essential facts i) These patients did not show a homogenous T-cell kinetic. Some of them had a static T-cell response characterized by the absence of T-cell mediated recognition for any of the latency antigens at both time points (fig. 21a, p. 69); others possessed increased amounts of IFNγ-expressing memory T cells at the later time point while T-cell responses for the latency antigens where next to detection limits at the beginning of treatment (fig. 21 b-d, p. 69). ii) There is also heterogeneity regarding the latency-associated antigens. In each individual TB patient with increased T-cell responses after sputum conversion a different subset of antigens was affected. Nevertheless the DosR encoded proteins Rv1734, Rv2003 and Rv2006 were consistently found among this subset (fig. 21 b-d, p. 69).
It is important to mention that in most patients the increase in T cell mediated recognition of the latency-associated antigens correlated with an increased IFNγ response towards the conventional immunodominant antigen ESAT6_CFP10 (fig. 21 c-d, p. 69). In conclusion the observed increase in T-cell recognition of the DosR encoded proteins could be due to a generally boosted T-cell response towards *M. tuberculosis* derived proteins along treatment. Death of mycobacteria and the presentation of their antigenic components might lead to increased antigen availability. Hence improved T-cell priming after prolonged administration of antibiotics could be an explanation for our observations. One might also

speculate that the increasing pressure by the combination of antibiotics and adaptive immune responses drive the pathogen into the dormant stages of its life cylce. The up-regulation of DosR encoded proteins could lead to the induction of specific $CD4^+$ T-cell responses. Some controversial reports regarding the kinetic of *M. tuberculosis*-specific immune responses along therapy have been discussed already (e.g. (Ewer, et al., 2006)). There are also some studies which documenter an elevated frequency of RD1-specific $CD4^+$ T cells at the beginning of treatment which levels off at later time points (Chee, et al., 2007; Veenstra, et al., 2007). Clearly this is in contrast with the results of this thesis.
Based on the present data it is impossible to claim which kind of T-cell response directed against which latency-associated antigen is really beneficial for the outcome of infection. Anyway it could be shown that TB patients (as well as LTBI) do not possess a homogenous T-cell response after *M. tuberculosis* infection and that there are fundamental differences in T-cell mediated recognition of latency-associated *M. tuberculosis* proteins. The cross-sectional comparison of LTBI and TB patients (fig. 17, p. 64) indicate that some latency-associated antigens are potential correlates of immunity to TB but this needs further evaluation.

Since childhood TB is often associated with severe courses of disease it is worth to study T-cell responses towards the pre-selected latency antigens in small children (below the age of six). The criteria for 'real' LTBI in these children is much more complicated compared to adults because infection with *M. tuberculosis* is treated immediately with antibiotics. This prophylactic treatment does not allow the identification of long-term resistant donors and blurs the boundaries between LTBI and active TB.
Indeed a situation different from that in adults could be observed; the preliminary study revealed T cell mediated recognition of all but one latency antigens in both study groups in the majority of tested individuals (fig. 22, p. 70). The only exception was Rv3407 which was exclusively recognized in LTBI and not in children with pulmonary TB. Interestingly the median of the amount of IFNγ expressing memory T cells for six of the latency-associated proteins was higher in TB patients than LTBI. Such a reverse situation compared to adults might be due to differences in the maturation stage of the immune system and/or antibiotic treatment. Perhaps the prophylactic therapy in children prevents a 'real' latency stage from

being evolved and consequently antigens associated with *M. tuberculosis* dormancy are not highly expressed.

7.3 Characterization of T-cell subsets

To further study the T-cell recognition of fractionated Beijing whole cell lysate, the associated cellular cytokine profiles were analyzed for each fraction. Therefore ICS followed by FACS analysis was used to detect either two or three cytokines simultaneously. This staining procedure allowed quantification of the amount of multi-functional T-cell proportions for each fraction. For the combination of IFNγ and TNFα no significant differences in the cytokine profiles were observed after restimulation of PBMC from donors with LTBI (fig. 25, p. 74). The vast majority of *M. tuberculosis*-specific $CD4^+$ $CD154^+$ T cells produced both cytokines in parallel and dissimilarities between the 11 fractions were absent. In contrast, patients with active TB had a reduced amount of IFNγ/TNFα dp T cells while the amount of $CD4^+$ $CD154^+$ T cells expressing neither IFNγ nor TNFα was increased. Even more each fraction elicited a slightly altered cytokine profile with different proportions of multi-functional T cells. This is an interesting finding since the amount of multi-functional T cells is thought to contribute to the outcome of viral and bacterial infections. It has been reported that the amount of $IFN\gamma^+$ $IL2^+$ $CD4^+$ T cells in HIV infected "Long-term-non-progressors" as well as individuals receiving anti-retroviral therapy is increased compared to progressors. The progressors predominantly had IFNγ sp T cells being specific for HIV encoded proteins (Boaz, et al., 2002; Harari, et al., 2004; Younes, et al., 2003). Similar results were found during chronic hepatitis C virus infection (Semmo, et al., 2005). More recent studies showed that a high amount of poly-functional T cells producing IFNγ and TNFα correlate with non-progressive HIV-2 infection and possibly a good clinical outcome (Duvall, et al., 2008). Also in cytomegalovirus infection extensive FACS analysis identified protective multi-functional $CD4^+$ T cells which produce MIP-1β, TNFα and IFNγ in the absence of IL2 (Casazza, et al., 2006). These $CD4^+$ T cells have a terminally differentiated phenotype and possess direct cytotoxic activity mediated by perforine and granzymes.

Remarkably, the degree of protection against *Leishmania major* infection in mice is predicted by the frequency of $CD4^+$ T cells simultaneously producing IFNγ, IL2 and TNFα.

Initial studies in humans revealed a shifted dominance to IFNγ/IL2 dp T cells during treatment of active TB (Millington, et al., 2007).

Conflicting to this report in the literature differences based on the expression of IFNγ/IL2 were absent while differences based on IFNγ/TNFα could be observed by this study (fig.26, p. 76). In contrast to this thesis, Millington *et. al.* used ESAT6_CFP10 for restimulatiuoin of T cells instead of *M. tuberculosis* lysate. Obviously the number of individuals tested for the expression of IFNγ/IL2 (n=3) and IFNg/TNFa (n=7) is too small to allow definitive statements. Additionally T-cell responses towards fractions can possibly lead to different results compared to those achieved by incubation with single immunogenic proteins (i.e. ESAT6_CFP10). Anyway the present findings emphasize the role of qualitative aspects of adaptive immune responses revealed by flow cytometry.

For further delineation of antigen-specific T-cell subsets recognizing fractionated Beijing lysate cytokine profiles of IFNγ, TNFα and IL2 were analyzed after restimulation (fig. 27, p. 77). This revealed a characteristic cytokine profile for each fraction with variable amounts of IFNγ/TNFα/IL2 tp and IFNγ/TNFα dp $CD4^+$ T cells. These findings accentuate the impact of multi-functional T-cell proportions and introduce a new aspect to our analysis, namely the quality of T-cell responses. However, the importance of these T-cell subsets *in vivo* as well as their impact on host defense against *M. tuberculosis* cannot be addressed by such experiments. Follow up studies of "Long-term-non-progressors" and progressors are needed to clarify this point. Since this kind of study is going beyond the scope of this PhD thesis a new functional *in vitro* assay was designed to evaluate the effects of different T-cell subsets on mycobacteria-infected macrophages. The results of this approach will be discussed later.

Beforehand it is worth to mention that low concentrations of IL7 were able to increase the amount of IFNγ/TNFα/IL2 tp and IFNγ/TNFα dp $CD4^+$ T cells after restimulation with Beijing whole cell lysate. For six patients with active pulmonary TB increased proportions of both T-cell subsets were observed (fig.29, p. 79). The responsiveness to IL7 seems to vary between donors leading to an increase of ~30% to ~120% for both T-cell sub-populations compared to the incubation with Beijing lysate alone. IL7 is a growth factor and continuously secreted by bone marrow stromal cells, thymic cells and keratinocytes. It is well known to promote survival of B cells (Melchers, et al., 1992), but it also plays an important role in T-cell homeostasis and maturation. The expression level of the associated

high affinity receptor (IL7Rα) has been proposed to contribute to T-cell expansion, contraction and memory development (Kaech, et al., 2003; Ma, et al., 2006).
Another theory suggests that deprivation of IL7 in combination with its limited availability account for these T-cell dynamics (Mazzucchelli and Durum, 2007).
The addition of recombinant IL7 at low amounts should optimize cell culture conditions for short-term restimulation of PBMC in this thesis. Especially a better T-cell survival and an improved responsiveness of $CD8^+$ T cells should be achieved by IL7. Surprisingly a specific effect of this cytokine on multi-functional T cells could be observed which gave rise to new questions. It has been reported that *in vitro* incubation with IL7 increases the production of T_h1 cytokines by mononuclear cells in patients with rheumatoid arthritis (van Roon, et al., 2003). Furthermore the administration of recombinant IL7 enhances survival of *M. tuberculosis* infected mice (Maeurer, et al., 2000). In animal experiments IL7 synergizes with IL15 and the transfer of spleenocytes from treated to untreated mice also increased survival (Maeurer, et al., 2000). All these hints together suggest an intertwined role of IL7 and multi-functional T cells. Since high effectiveness is crucial for the success of vaccination strategies the generation of a recombinant BCG vaccine expressing IL7 could be a promising approach.

7.4 Functional *in vitro* assays

To address the relevance of IL7 and functional aspects of different T-cell subsets, novel *in vitro* assays are needed. They should allow rapid quantification of viable intracellular mycobacteria as well as assessment of T cell/macrophage interactions. Especially the contribution of different T-cell subset to killing of mycobacteria should be measureable. Monocyte-derived macrophages were used as target cells for infection with eGFP expressing mycobacteria since they are very similar to the primary host cell of *M. tuberculosis in vivo*. Macrophage-like cell lines were no alternative due to the use of autologous PBMC as effector cells. The conventional method for determination of bacterial titers is plating bacteria on agar medium and counting of CFU. This method is on the one hand error-prone and on the other hand incapable of providing information about side damage to host macrophages. Some novel assays have been established to gain insights into direct killing mechanisms of T cells (Snyder, et al., 2003). None of these approaches is able to provide

information on survival of target cells and killing of mycobacteria simultaneously. The direct FACS analysis of $eGFP^+$ macrophages in combination with staining for lineage markers allows quantification of vital macrophages and determination of bacterial burden in parallel.

As an initial step to address the feasibility of the *in vitro* killing assay we compared the results gained by flow cytometry to conventional determination of CFU on plates. Therefore BCG infected monocyte-derived macrophages were incubated with different concentrations of rifampicine and streptomycin, respectively (fig. 33, p. 84). A dose dependent reduction of the bacterial load could be observed by flow cytometry and CFU after treatment with both antibiotics. Interestingly the FACS approach detected generally higher bacterial titers after incubation with antibiotics compared to CFU. Probably this is due to long-term effects after treatment with antibiotics which inhibit the growth on plates and cannot be observed immediately after cell culture. Nevertheless the dose dependent reduction of intracellular mycobacteria was detectable by flow cytometry and the new method seems to be less error-prone than conventional CFU. Since killing by autologous effector PBMC is likely to be a direct effect (Stenger, 2001), the dissimilarity of FACS and CFU regarding bacteriostatic effects will be irrelevant for our questions. Hence the flow cytometry-based assay provides direct and reliable information about immediate killing of intracellular mycobacteria.

Consequently infected macrophages were co-cultured with PBMC activated by *M. tuberculosis* lysate and unstimulated PBMC, respectively (fig.34, p. 86). The incubation with unstimulated PBMC (PBMC/macrophage ratio = 10:1) already reduced the amount of intracellular bacteria. Notably the bactericidal effect was much stronger if PBMC activated by *M. tuberculosis* lysate were used. The same tendency was observed by FACS analysis and CFU, but in contrast to flow cytometry the CFU data suffered from a higher standard error which impaired the ability to detect minor changes in bacterial titers. Similar results regarding the direct or indirect killing effect of autologous PBMC were observed within independent experiments using PBMC from another donor (fig. 32, p. 83). The detection of $eGFP^+$ macrophages by flow cytometry documented the killing effect of autologous PBMC activated by *M. tuberculosis* lysate. The killing effect was even more pronounced for IL2 stimulated LAK cells (fig. 32, p. 83).

In order to show that autologous effector PBMC decrease the bacterial load in a dose-dependent manner different amounts of PBMC were added to infected macrophages (fig. 35,

p. 87). Initially an effector/target ratio of 5-1 and 10-1 was applied. To get an impression about the killing mechanism the ratio of $CD4^+$ T cells and $CD11b^+$ macrophages was calculated at the end of the assay. Since the amount of T cells remains constant during the co-culture period an increase in the final effector/target ratio measured by flow cytometry indicates killing of macrophages (for further details see *6.4.1 Methodology,* p. 80). This allows a quantification of side damage to macrophages and is a unique feature of the novel assay. In comparison to unstimulated PBMC, FACS and CFU both documented a dose-dependent reduction of intracellular mycobacteria by autologous PBMC activated by *M. tuberculosis* or IL2. The strength of the killing effect was comparable and high amounts of lysate-activated PBMC were even more potent than LAK cells. Both methods seem to lead to identical results at first glance. Taking the calculation of final effector/target ratios into account, more detailed information was gained by FACS analysis than by CFU alone. It turned out that PBMC stimulated by *M. tuberculosis* lysate caused slight side damage to infected macrophages while LAK cells killed significantly more macrophages under the same conditions. Consequently the inclusion of modern flow cytometry provided valuable information about killing mechanisms and revealed qualitative differences.

These findings demonstrated the potential of lysate-activated PBMC to reduce the bacterial burden without massive side damage to macrophages. The observed killing effect was as strong as the effect mediated by LAK cells. Discrimination between both killing mechanisms was possible since LAK cells caused significant side damage to macrophages which was not the case for lysate-activated PBMC. Remarkably, increasing amounts of effector PBMC led to an increased killing of mycobacteria and IL2 concentrations above 10U/ml were needed to induce LAK cells.

Detailed information about the underlying molecular mechanisms of LAK cells and lysate activated PBMC are rare in the literature. The generation of LAK cells by high dose treatment with recombinant IL2 induces more unspecific ways of killing carried out mainly by NK cells (Hiserodt, 1993). It is also known that IL2 acts as an antigen-unspecific proliferation factor for T cells and allows clonal expansion (Burchill, et al., 2007). Although IL2 activated LAK cells were extensively used for adoptive immunotherapy against cancer in the past, the molecular basis of the interactions between LAK cells and their target cells has not yet been elucidated in detail. The involvement of Fc-receptors on LAK cells

suggests antibody-dependent cytotoxicity, but perforin as well as granzymes also seem to play a role (Hank, et al., 1988).

The killing mechanism mediated by *M. tuberculosis* lysate-activated PBMC remains elusive, as well. Two possible explanations may account for the killing effects of these cells: i) MHC restricted recognition of conventional peptides or unconventional (glycol-) lipids presented via CD1d; ii) nonspecific activation of PBMC, e.g. by residual TLR ligands within the lysate. To distinguish between both mechanisms a block of MHC-I and MHC-II had been performed by antibodies. Unfortunately the effector/target co-culture period of 36 hours was too long for such blocking experiments and significantly reduced survival of effector PBMC (data not shown). Alternatively different T-cell populations were isolated and applied separately to the infected macrophages. Using the MACS technology $CD4^+$ and $CD8^+$ T cells as well as $CD56^+$ NK cells were purified after stimulation with *M. tuberculosis* lysate. $CD56^+$ NK cells did not have a stronger killing effect than unstimulated PBMC but the results for $CD4^+$ and $CD8^+$ T cells were less clear (data not shown). Consequently *M. tuberculosis* lysate was used as a surrogate stimulus during the initial phase of this assay which can be replaced by recombinant immunodominant proteins of *M. tuberculosis* like TB10.4 or Ag85a/b in the future.

Considering the whole set of performed experiments, there are some crucial points of criticism which impair the potential of this novel assay. For all experiments based on antibiotics the direct quantification of $eGFP^+$ macrophages by flow cytometry correlated with determination of CFU on plates (fig.37, p. 90). In contrast, among all eight experiments based on autologous PBMC only six showed a correlation of FACS analysis and CFU data. In some cases killing of bacteria was detected by measuring $eGFP^+$ macrophages which could not be verified by CFU and vice versa. Moreover for the same donor and identical culture conditions killing by autologous effector PBMC could be measured in some cases and not in others (donor 083; fig. 37, p. 90). Possibly the generation of monocyte-derived macrophages lead to a heterogeneous target cell population which was more or less susceptible for infection with mycobacteria. An example is the variable proportion of CD16 expressing macrophages among the target cell population. Such assay to assay variations could be misleading and interfere with the evaluation of T-cell help.

Similar problems were observed by other groups working on functional *in vitro* assays (Prof. S. Stenger, *personal communication*). The exchange of monocyte-derived macrophages by end-differentiated alveolar macrophages may be a possible solution. These lung-resident macrophages can be obtained by bronchoalveolar lavage, but they are only present in low absolute numbers in these samples. Ethical questions may also arise since this kind of medical examination is not free of risk and needs a therapeutic indication. Additionally it would be difficult to collect peripheral blood form a patient in the clinic 7d prior to the lavage in order to generate autologous effects cells.

Despite all difficulties, the dose-dependent decrease in bacterial burden after treatment with antibiotics proof the potential of this assay to directly assess the level of intracellular bacteria. In combination with conventional CFU, the FACS based analysis provides important information about the quality of the observed killing, which cannot be addressed by bacterial growth analysis alone. Therefore it helps to elucidate different killing mechanisms. On the other hand, assay to assay variations as well as high technical requirements (e.g. the need of FACS analyzers and equipment for cell culture) designates the assay for academic research but limits its applicability for field studies in developing countries.

8 References

Ackerman, A. L. and Cresswell, P. (2004): Cellular mechanisms governing cross-presentation of exogenous antigens, Nat Immunol 5 [7], pp. 678-84.

Acosta-Rodriguez, E. V.; Napolitani, G.; Lanzavecchia, A. and Sallusto, F. (2007): Interleukins 1beta and 6 but not transforming growth factor-beta are essential for the differentiation of interleukin 17-producing human T helper cells, Nat Immunol 8 [9], pp. 942-9.

Aderem, A. and Underhill, D. M. (1999): Mechanisms of phagocytosis in macrophages, Annu Rev Immunol 17, pp. 593-623.

Aggarwal, S.; Ghilardi, N.; Xie, M. H.; de Sauvage, F. J. and Gurney, A. L. (2003): Interleukin-23 promotes a distinct CD4 T cell activation state characterized by the production of interleukin-17, J Biol Chem 278 [3], pp. 1910-4.

Alderson, M. R.; Sassenfeld, H. M. and Widmer, M. B. (1990): Interleukin 7 enhances cytolytic T lymphocyte generation and induces lymphokine-activated killer cells from human peripheral blood, J Exp Med 172 [2], pp. 577-87.

Alsharifi, M.; Mullbacher, A. and Regner, M. (2008): Interferon type I responses in primary and secondary infections, Immunol Cell Biol 86 [3], pp. 239-45.

Annacker, O.; Pimenta-Araujo, R.; Burlen-Defranoux, O.; Barbosa, T. C.; Cumano, A. and Bandeira, A. (2001): CD25+ CD4+ T cells regulate the expansion of peripheral CD4 T cells through the production of IL-10, J Immunol 166 [5], pp. 3008-18.

Arend, S. M.; Andersen, P.; van Meijgaarden, K. E.; Skjot, R. L.; Subronto, Y. W.; van Dissel, J. T. and Ottenhoff, T. H. (2000): Detection of active tuberculosis infection by T cell responses to early-secreted antigenic target 6-kDa protein and culture filtrate protein 10, J Infect Dis 181 [5], pp. 1850-4.

Arend, S. M.; Thijsen, S. F.; Leyten, E. M.; Bouwman, J. J.; Franken, W. P.; Koster, B. F.; Cobelens, F. G.; van Houte, A. J. and Bossink, A. W. (2007): Comparison of two interferon-gamma assays and tuberculin skin test for tracing tuberculosis contacts, Am J Respir Crit Care Med 175 [6], pp. 618-27.

Armitage, R. J.; Ziegler, S. F.; Friend, D. J.; Park, L. S. and Fanslow, W. C. (1992): Identification of a novel low-affinity receptor for human interleukin-7, Blood 79 [7], pp. 1738-45.

Asensio, J. A.; Arbues, A.; Perez, E.; Gicquel, B. and Martin, C. (2008): Live tuberculosis vaccines based on phoP mutants: a step towards clinical trials, Expert Opin Biol Ther 8 [2], pp. 201-11.

Baldwin, A. S., Jr. (1996): The NF-kappa B and I kappa B proteins: new discoveries and insights, Annu Rev Immunol 14, pp. 649-83.

Barry, C. E.; Crick, D. C. and McNeil, M. R. (2007): Targeting the formation of the cell wall core of M. tuberculosis, Infect Disord Drug Targets 7 [2], pp. 182-202.

Barry, M. and Bleackley, R. C. (2002): Cytotoxic T lymphocytes: all roads lead to death, Nat Rev Immunol 2 [6], pp. 401-9.

Bellamy, R.; Ruwende, C.; Corrah, T.; McAdam, K. P.; Whittle, H. C. and Hill, A. V. (1998): Assessment of the interleukin 1 gene cluster and other candidate gene polymorphisms in host susceptibility to tuberculosis, Tuber Lung Dis 79 [2], pp. 83-9.

Berthet, F. X.; Rasmussen, P. B.; Rosenkrands, I.; Andersen, P. and Gicquel, B. (1998): A Mycobacterium tuberculosis operon encoding ESAT-6 and a novel low-molecular-mass culture filtrate protein (CFP-10), Microbiology 144 (Pt 11), pp. 3195-203.

Bevan, M. J. (2004): Helping the CD8(+) T-cell response, Nat Rev Immunol 4 [8], pp. 595-602.

Biketov, S.; Mukamolova, G. V.; Potapov, V.; Gilenkov, E.; Vostroknutova, G.; Kell, D. B.; Young, M. and Kaprelyants, A. S. (2000): Culturability of Mycobacterium tuberculosis cells isolated from murine macrophages: a bacterial growth factor promotes recovery, FEMS Immunol Med Microbiol 29 [4], pp. 233-40.

Blackwell, J. M.; Searle, S.; Goswami, T. and Miller, E. N. (2000): Understanding the multiple functions of Nramp1, Microbes Infect 2 [3], pp. 317-21.

Boaz, M. J.; Waters, A.; Murad, S.; Easterbrook, P. J. and Vyakarnam, A. (2002): Presence of HIV-1 Gag-specific IFN-gamma+IL-2+ and CD28+IL-2+ CD4 T cell responses is associated with nonprogression in HIV-1 infection, J Immunol 169 [11], pp. 6376-85.

Boehm, U.; Klamp, T.; Groot, M. and Howard, J. C. (1997): Cellular responses to interferon-gamma, Annu Rev Immunol 15, pp. 749-95.

Bogdan, C.; Rollinghoff, M. and Diefenbach, A. (2000): Reactive oxygen and reactive nitrogen intermediates in innate and specific immunity, Curr Opin Immunol 12 [1], pp. 64-76.

Boon, C. and Dick, T. (2002): Mycobacterium bovis BCG response regulator essential for hypoxic dormancy, J Bacteriol 184 [24], pp. 6760-7.

Bothamley, G. H. (1999): Differences between HLA-B44 and HLA-B60 in patients with smear-positive pulmonary tuberculosis and exposed controls, J Infect Dis 179 [4], pp. 1051-2.

Bothamley, G. H.; Beck, J. S.; Schreuder, G. M.; D'Amaro, J.; de Vries, R. R.; Kardjito, T. and Ivanyi, J. (1989): Association of tuberculosis and M. tuberculosis-specific antibody levels with HLA, J Infect Dis 159 [3], pp. 549-55.

Bowdish, D. M.; Loffredo, M. S.; Mukhopadhyay, S.; Mantovani, A. and Gordon, S. (2007): Macrophage receptors implicated in the "adaptive" form of innate immunity, Microbes Infect 9 [14-15], pp. 1680-7.

Boztug, K.; Carson, M. J.; Pham-Mitchell, N.; Asensio, V. C.; DeMartino, J. and Campbell, I. L. (2002): Leukocyte infiltration, but not neurodegeneration, in the CNS of transgenic mice with astrocyte production of the CXC chemokine ligand 10, J Immunol 169 [3], pp. 1505-15.

Brassard, P.; Kezouh, A. and Suissa, S. (2006): Antirheumatic drugs and the risk of tuberculosis, Clin Infect Dis 43 [6], pp. 717-22.

Breiman, L. (2001): Random Forests., Machine Learning 45 [1], pp. 5-32.

Britton, W. J.; Fernando, S. L.; Saunders, B. M.; Sluyter, R. and Wiley, J. S. (2007): The genetic control of susceptibility to Mycobacterium tuberculosis, Novartis Found Symp 281, pp. 79-89; discussion 89-92, 208-9.

Brock, T. D. (1988): Robert Koch, Springer Verlag.

Bryk, R.; Lima, C. D.; Erdjument-Bromage, H.; Tempst, P. and Nathan, C. (2002): Metabolic enzymes of mycobacteria linked to antioxidant defense by a thioredoxin-like protein, Science 295 [5557], pp. 1073-7.

Burchill, M. A.; Yang, J.; Vang, K. B. and Farrar, M. A. (2007): Interleukin-2 receptor signaling in regulatory T cell development and homeostasis, Immunol Lett 114 [1], pp. 1-8.

Burgdorf, S.; Scholz, C.; Kautz, A.; Tampe, R. and Kurts, C. (2008): Spatial and mechanistic separation of cross-presentation and endogenous antigen presentation, Nat Immunol.

Bustamante, J.; Picard, C.; Fieschi, C.; Filipe-Santos, O.; Feinberg, J.; Perronne, C.; Chapgier, A.; de Beaucoudrey, L.; Vogt, G.; Sanlaville, D.; Lemainque, A.; Emile, J. F.; Abel, L. and Casanova, J. L. (2007): A novel X-linked recessive form of Mendelian susceptibility to mycobaterial disease, J Med Genet 44 [2], p. e65.

Caruso, A. M.; Serbina, N.; Klein, E.; Triebold, K.; Bloom, B. R. and Flynn, J. L. (1999): Mice deficient in CD4 T cells have only transiently diminished levels of IFN-gamma, yet succumb to tuberculosis, J Immunol 162 [9], pp. 5407-16.

Casazza, J. P.; Betts, M. R.; Price, D. A.; Precopio, M. L.; Ruff, L. E.; Brenchley, J. M.; Hill, B. J.; Roederer, M.; Douek, D. C. and Koup, R. A. (2006): Acquisition of direct antiviral effector functions by CMV-specific CD4+ T lymphocytes with cellular maturation, J Exp Med 203 [13], pp. 2865-77.

Cehovin, A.; Cliff, J. M.; Hill, P. C.; Brookes, R. H. and Dockrell, H. M. (2007): Extended culture enhances sensitivity of a gamma interferon assay for latent Mycobacterium tuberculosis infection, Clin Vaccine Immunol 14 [6], pp. 796-8.

Chan, J. and Flynn, J. (2004): The immunological aspects of latency in tuberculosis, Clin Immunol 110 [1], pp. 2-12.

Chee, C. B.; KhinMar, K. W.; Gan, S. H.; Barkham, T. M.; Pushparani, M. and Wang, Y. T. (2007): Latent tuberculosis infection treatment and T-cell responses to Mycobacterium tuberculosis-specific antigens, Am J Respir Crit Care Med 175 [3], pp. 282-7.

Cherwinski, H. M.; Schumacher, J. H.; Brown, K. D. and Mosmann, T. R. (1987): Two types of mouse helper T cell clone. III. Further differences in lymphokine synthesis between Th1 and Th2 clones revealed by RNA hybridization, functionally monospecific bioassays, and monoclonal antibodies, J Exp Med 166 [5], pp. 1229-44.

Clarke, A. and Rudd, P. (1992): Neonatal BCG immunisation, Arch Dis Child 67 [4], pp. 473-4.

Cohen-Gonsaud, M.; Barthe, P.; Bagneris, C.; Henderson, B.; Ward, J.; Roumestand, C. and Keep, N. H. (2005): The structure of a resuscitation-promoting factor domain from Mycobacterium tuberculosis shows homology to lysozymes, Nat Struct Mol Biol 12 [3], pp. 270-3.

Cole, S. T.; Brosch, R.; Parkhill, J.; Garnier, T.; Churcher, C.; Harris, D.; Gordon, S. V.; Eiglmeier, K.; Gas, S.; Barry, C. E., 3rd; Tekaia, F.; Badcock, K.; Basham, D.; Brown, D.; Chillingworth, T.; Connor, R.; Davies, R.; Devlin, K.; Feltwell, T.; Gentles, S.; Hamlin, N.; Holroyd, S.; Hornsby, T.; Jagels, K.; Krogh, A.; McLean, J.; Moule, S.; Murphy, L.; Oliver, K.; Osborne, J.; Quail, M. A.; Rajandream, M. A.; Rogers, J.; Rutter, S.; Seeger, K.; Skelton, J.; Squares, R.; Squares, S.; Sulston, J. E.; Taylor, K.; Whitehead, S. and Barrell, B. G. (1998): Deciphering the biology of Mycobacterium tuberculosis from the complete genome sequence, Nature 393 [6685], pp. 537-44.

Cooper, A. M.; Dalton, D. K.; Stewart, T. A.; Griffin, J. P.; Russell, D. G. and Orme, I. M. (1993): Disseminated tuberculosis in interferon gamma gene-disrupted mice, J Exp Med 178 [6], pp. 2243-7.

Cunningham, A. F. and Spreadbury, C. L. (1998): Mycobacterial stationary phase induced by low oxygen tension: cell wall thickening and localization of the 16-kilodalton alpha-crystallin homolog, J Bacteriol 180 [4], pp. 801-8.

de Jonge, M. I.; Pehau-Arnaudet, G.; Fretz, M. M.; Romain, F.; Bottai, D.; Brodin, P.; Honore, N.; Marchal, G.; Jiskoot, W.; England, P.; Cole, S. T. and Brosch, R. (2007): ESAT-6 from Mycobacterium tuberculosis dissociates from its putative chaperone

CFP-10 under acidic conditions and exhibits membrane-lysing activity, J Bacteriol 189 [16], pp. 6028-34.

Delves, P. J. and Roitt, I. M. (2000): The immune system. First of two parts, N Engl J Med 343 [1], pp. 37-49.

Dietrich, J.; Aagaard, C.; Leah, R.; Olsen, A. W.; Stryhn, A.; Doherty, T. M. and Andersen, P. (2005): Exchanging ESAT6 with TB10.4 in an Ag85B fusion molecule-based tuberculosis subunit vaccine: efficient protection and ESAT6-based sensitive monitoring of vaccine efficacy, J Immunol 174 [10], pp. 6332-9.

Duerr, R. H.; Taylor, K. D.; Brant, S. R.; Rioux, J. D.; Silverberg, M. S.; Daly, M. J.; Steinhart, A. H.; Abraham, C.; Regueiro, M.; Griffiths, A.; Dassopoulos, T.; Bitton, A.; Yang, H.; Targan, S.; Datta, L. W.; Kistner, E. O.; Schumm, L. P.; Lee, A. T.; Gregersen, P. K.; Barmada, M. M.; Rotter, J. I.; Nicolae, D. L. and Cho, J. H. (2006): A genome-wide association study identifies IL23R as an inflammatory bowel disease gene, Science 314 [5804], pp. 1461-3.

Dufour, J. H.; Dziejman, M.; Liu, M. T.; Leung, J. H.; Lane, T. E. and Luster, A. D. (2002): IFN-gamma-inducible protein 10 (IP-10; CXCL10)-deficient mice reveal a role for IP-10 in effector T cell generation and trafficking, J Immunol 168 [7], pp. 3195-204.

Duvall, M. G.; Precopio, M. L.; Ambrozak, D. A.; Jaye, A.; McMichael, A. J.; Whittle, H. C.; Roederer, M.; Rowland-Jones, S. L. and Koup, R. A. (2008): Polyfunctional T cell responses are a hallmark of HIV-2 infection, Eur J Immunol 38 [2], pp. 350-63.

Ehrt, S.; Shiloh, M. U.; Ruan, J.; Choi, M.; Gunzburg, S.; Nathan, C.; Xie, Q. and Riley, L. W. (1997): A novel antioxidant gene from Mycobacterium tuberculosis, J Exp Med 186 [11], pp. 1885-96.

Ewer, K.; Millington, K. A.; Deeks, J. J.; Alvarez, L.; Bryant, G. and Lalvani, A. (2006): Dynamic antigen-specific T-cell responses after point-source exposure to Mycobacterium tuberculosis, Am J Respir Crit Care Med 174 [7], pp. 831-9.

Feili-Hariri, M.; Falkner, D. H. and Morel, P. A. (2005): Polarization of naive T cells into Th1 or Th2 by distinct cytokine-driven murine dendritic cell populations: implications for immunotherapy, J Leukoc Biol 78 [3], pp. 656-64.

Fernando, S. L. and Britton, W. J. (2006): Genetic susceptibility to mycobacterial disease in humans, Immunol Cell Biol 84 [2], pp. 125-37.

Ferrari, G.; Langen, H.; Naito, M. and Pieters, J. (1999): A coat protein on phagosomes involved in the intracellular survival of mycobacteria, Cell 97 [4], pp. 435-47.

Fontenot, J. D.; Gavin, M. A. and Rudensky, A. Y. (2003): Foxp3 programs the development and function of CD4+CD25+ regulatory T cells, Nat Immunol 4 [4], pp. 330-6.

Foxwell, B. M.; Barrett, K. and Feldmann, M. (1992): Cytokine receptors: structure and signal transduction, Clin Exp Immunol 90 [2], pp. 161-9.

Goldfeld, A. E.; Delgado, J. C.; Thim, S.; Bozon, M. V.; Uglialoro, A. M.; Turbay, D.; Cohen, C. and Yunis, E. J. (1998): Association of an HLA-DQ allele with clinical tuberculosis, Jama 279 [3], pp. 226-8.

Graham, J. E. and Clark-Curtiss, J. E. (1999): Identification of Mycobacterium tuberculosis RNAs synthesized in response to phagocytosis by human macrophages by selective capture of transcribed sequences (SCOTS), Proc Natl Acad Sci U S A 96 [20], pp. 11554-9.

Grode, L.; Seiler, P.; Baumann, S.; Hess, J.; Brinkmann, V.; Nasser Eddine, A.; Mann, P.; Goosmann, C.; Bandermann, S.; Smith, D.; Bancroft, G. J.; Reyrat, J. M.; van Soolingen, D.; Raupach, B. and Kaufmann, S. H. (2005): Increased vaccine efficacy

against tuberculosis of recombinant Mycobacterium bovis bacille Calmette-Guerin mutants that secrete listeriolysin, J Clin Invest 115 [9], pp. 2472-9.

Guermonprez, P.; Saveanu, L.; Kleijmeer, M.; Davoust, J.; Van Endert, P. and Amigorena, S. (2003): ER-phagosome fusion defines an MHC class I cross-presentation compartment in dendritic cells, Nature 425 [6956], pp. 397-402.

Guevara-Patino, J. A.; Turk, M. J.; Wolchok, J. D. and Houghton, A. N. (2003): Immunity to cancer through immune recognition of altered self: studies with melanoma, Adv Cancer Res 90, pp. 157-77.

Guimond, M.; Fry, T. J. and Mackall, C. L. (2005): Cytokine signals in T-cell homeostasis, J Immunother 28 [4], pp. 289-94.

Hank, J. A.; Kohler, P. C.; Weil-Hillman, G.; Rosenthal, N.; Moore, K. H.; Storer, B.; Minkoff, D.; Bradshaw, J.; Bechhofer, R. and Sondel, P. M. (1988): In vivo induction of the lymphokine-activated killer phenomenon: interleukin 2-dependent human non-major histocompatibility complex-restricted cytotoxicity generated in vivo during administration of human recombinant interleukin 2, Cancer Res 48 [7], pp. 1965-71.

Harari, A.; Petitpierre, S.; Vallelian, F. and Pantaleo, G. (2004): Skewed representation of functionally distinct populations of virus-specific CD4 T cells in HIV-1-infected subjects with progressive disease: changes after antiretroviral therapy, Blood 103 [3], pp. 966-72.

Hart, P. D.; Armstrong, J. A.; Brown, C. A. and Draper, P. (1972): Ultrastructural study of the behavior of macrophages toward parasitic mycobacteria, Infect Immun 5 [5], pp. 803-7.

Heath, W. R. and Carbone, F. R. (2001): Cross-presentation in viral immunity and self-tolerance, Nat Rev Immunol 1 [2], pp. 126-34.

Hirsch, C. S.; Toossi, Z.; Othieno, C.; Johnson, J. L.; Schwander, S. K.; Robertson, S.; Wallis, R. S.; Edmonds, K.; Okwera, A.; Mugerwa, R.; Peters, P. and Ellner, J. J. (1999): Depressed T-cell interferon-gamma responses in pulmonary tuberculosis: analysis of underlying mechanisms and modulation with therapy, J Infect Dis 180 [6], pp. 2069-73.

Hiserodt, J. C. (1993): Lymphokine-activated killer cells: biology and relevance to disease, Cancer Invest 11 [4], pp. 420-39.

Hori, S.; Nomura, T. and Sakaguchi, S. (2003): Control of regulatory T cell development by the transcription factor Foxp3, Science 299 [5609], pp. 1057-61.

Horwitz, M. A. and Harth, G. (2003): A new vaccine against tuberculosis affords greater survival after challenge than the current vaccine in the guinea pig model of pulmonary tuberculosis, Infect Immun 71 [4], pp. 1672-9.

Hosken, N. A.; Bevan, M. J. and Carbone, F. R. (1989): Class I-restricted presentation occurs without internalization or processing of exogenous antigenic peptides, J Immunol 142 [4], pp. 1079-83.

Jacobsen, M.; Mattow, J.; Repsilber, D. and Kaufmann, S. H. (2008): Novel strategies to identify biomarkers in tuberculosis, Biol Chem.

Janeway, C. A. (2005): Immunobiology, 6th. ed., ISBN: 0-8153-4101-6.

Jankovic, D.; Liu, Z. and Gause, W. C. (2001): Th1- and Th2-cell commitment during infectious disease: asymmetry in divergent pathways, Trends Immunol 22 [8], pp. 450-7.

Jo, E. K. (2008): Mycobacterial interaction with innate receptors: TLRs, C-type lectins, and NLRs, Curr Opin Infect Dis 21 [3], pp. 279-86.

Jouanguy, E.; Lamhamedi-Cherradi, S.; Altare, F.; Fondaneche, M. C.; Tuerlinckx, D.; Blanche, S.; Emile, J. F.; Gaillard, J. L.; Schreiber, R.; Levin, M.; Fischer, A.; Hivroz, C. and Casanova, J. L. (1997): Partial interferon-gamma receptor 1 deficiency in a child with tuberculoid bacillus Calmette-Guerin infection and a sibling with clinical tuberculosis, J Clin Invest 100 [11], pp. 2658-64.

Kaech, S. M.; Tan, J. T.; Wherry, E. J.; Konieczny, B. T.; Surh, C. D. and Ahmed, R. (2003): Selective expression of the interleukin 7 receptor identifies effector CD8 T cells that give rise to long-lived memory cells, Nat Immunol 4 [12], pp. 1191-8.

Kahnert, A.; Hopken, U. E.; Stein, M.; Bandermann, S.; Lipp, M. and Kaufmann, S. H. (2007): Mycobacterium tuberculosis triggers formation of lymphoid structure in murine lungs, J Infect Dis 195 [1], pp. 46-54.

Kana, B. D.; Gordhan, B. G.; Downing, K. J.; Sung, N.; Vostroktunova, G.; Machowski, E. E.; Tsenova, L.; Young, M.; Kaprelyants, A.; Kaplan, G. and Mizrahi, V. (2008): The resuscitation-promoting factors of Mycobacterium tuberculosis are required for virulence and resuscitation from dormancy but are collectively dispensable for growth in vitro, Mol Microbiol 67 [3], pp. 672-84.

Kaufmann, S. H. and McMichael, A. J. (2005): Annulling a dangerous liaison: vaccination strategies against AIDS and tuberculosis, Nat Med 11 [4 Suppl], pp. S33-44.

Kawamura, I. (2006): [Protective immunity against Mycobacterium tuberculosis], Kekkaku 81 [11], pp. 687-91.

Keep, N. H.; Ward, J. M.; Cohen-Gonsaud, M. and Henderson, B. (2006): Wake up! Peptidoglycan lysis and bacterial non-growth states, Trends Microbiol 14 [6], pp. 271-6.

Khader, S. A.; Bell, G. K.; Pearl, J. E.; Fountain, J. J.; Rangel-Moreno, J.; Cilley, G. E.; Shen, F.; Eaton, S. M.; Gaffen, S. L.; Swain, S. L.; Locksley, R. M.; Haynes, L.; Randall, T. D. and Cooper, A. M. (2007): IL-23 and IL-17 in the establishment of protective pulmonary CD4+ T cell responses after vaccination and during Mycobacterium tuberculosis challenge, Nat Immunol 8 [4], pp. 369-77.

Khader, S. A. and Cooper, A. M. (2008): IL-23 and IL-17 in tuberculosis, Cytokine 41 [2], pp. 79-83.

Kimiyoshi, T., Aizawa, M., Sasazuki,T. (1991): Proceedings of the Eleventh International Histocompatibility Workshop and Conference Held in Yokohoma, Japan, 6-13 November, 1991., Oxford University Press, Oxford, ISBN: 0-19-262390-7.

Kloetzel, P. M. (2001): Antigen processing by the proteasome, Nat Rev Mol Cell Biol 2 [3], pp. 179-87.

Koch, R (1882): Die Aetiologie der Tuberculose, Berliner Klinische Wochenschrift 19 [15].

Kolls, J. K. and Linden, A. (2004): Interleukin-17 family members and inflammation, Immunity 21 [4], pp. 467-76.

Krammer, P. H.; Arnold, R. and Lavrik, I. N. (2007): Life and death in peripheral T cells, Nat Rev Immunol 7 [7], pp. 532-42.

Lalvani, A. (2007): Diagnosing tuberculosis infection in the 21st century: new tools to tackle an old enemy, Chest 131 [6], pp. 1898-906.

Lehmann, KB and Neumann, RO (1896): Atlas und Grundriss der Bakteriologie und Lehrbuch der speciellen bakteriologischen Diagnostik.

Levin, M. and Newport, M. (1999): Understanding the genetic basis of susceptibility to mycobacterial infection, Proc Assoc Am Physicians 111 [4], pp. 308-12.

Leyten, E. M.; Arend, S. M.; Prins, C.; Cobelens, F. G.; Ottenhoff, T. H. and van Dissel, J. T. (2007): Discrepancy between Mycobacterium tuberculosis-specific gamma interferon

release assays using short and prolonged in vitro incubation, Clin Vaccine Immunol 14 [7], pp. 880-5.

Leyten, E. M.; Lin, M. Y.; Franken, K. L.; Friggen, A. H.; Prins, C.; van Meijgaarden, K. E.; Voskuil, M. I.; Weldingh, K.; Andersen, P.; Schoolnik, G. K.; Arend, S. M.; Ottenhoff, T. H. and Klein, M. R. (2006): Human T-cell responses to 25 novel antigens encoded by genes of the dormancy regulon of Mycobacterium tuberculosis, Microbes Infect 8 [8], pp. 2052-60.

Ma, A.; Koka, R. and Burkett, P. (2006): Diverse functions of IL-2, IL-15, and IL-7 in lymphoid homeostasis, Annu Rev Immunol 24, pp. 657-79.

Maecker, H. T.; Dunn, H. S.; Suni, M. A.; Khatamzas, E.; Pitcher, C. J.; Bunde, T.; Persaud, N.; Trigona, W.; Fu, T. M.; Sinclair, E.; Bredt, B. M.; McCune, J. M.; Maino, V. C.; Kern, F. and Picker, L. J. (2001): Use of overlapping peptide mixtures as antigens for cytokine flow cytometry, J Immunol Methods 255 [1-2], pp. 27-40.

Maeurer, M. J.; Trinder, P.; Hommel, G.; Walter, W.; Freitag, K.; Atkins, D. and Storkel, S. (2000): Interleukin-7 or interleukin-15 enhances survival of Mycobacterium tuberculosis-infected mice, Infect Immun 68 [5], pp. 2962-70.

Maglione, P. J.; Xu, J. and Chan, J. (2007): B cells moderate inflammatory progression and enhance bacterial containment upon pulmonary challenge with Mycobacterium tuberculosis, J Immunol 178 [11], pp. 7222-34.

Masson, D.; Zamai, M. and Tschopp, J. (1986): Identification of granzyme A isolated from cytotoxic T-lymphocyte-granules as one of the proteases encoded by CTL-specific genes, FEBS Lett 208 [1], pp. 84-8.

Maziak, W. (2003): The Th1-Th2 paradigm and asthma: how far should we go?, J Asthma 40 [2], pp. 201-5.

Mazzucchelli, R. and Durum, S. K. (2007): Interleukin-7 receptor expression: intelligent design, Nat Rev Immunol 7 [2], pp. 144-54.

McShane, H.; Pathan, A. A.; Sander, C. R.; Keating, S. M.; Gilbert, S. C.; Huygen, K.; Fletcher, H. A. and Hill, A. V. (2004): Recombinant modified vaccinia virus Ankara expressing antigen 85A boosts BCG-primed and naturally acquired antimycobacterial immunity in humans, Nat Med 10 [11], pp. 1240-4.

Means, T. K.; Wang, S.; Lien, E.; Yoshimura, A.; Golenbock, D. T. and Fenton, M. J. (1999): Human toll-like receptors mediate cellular activation by Mycobacterium tuberculosis, J Immunol 163 [7], pp. 3920-7.

Medzhitov, R. (2007): Recognition of microorganisms and activation of the immune response, Nature 449 [7164], pp. 819-26.

Medzhitov, R. and Janeway, C., Jr. (2000): Innate immunity, N Engl J Med 343 [5], pp. 338-44.

Melchers, F.; Haasner, D.; Streb, M. and Rolink, A. (1992): B-lymphocyte lineage-committed, IL-7 and stroma cell-reactive progenitors and precursors, and their differentiation to B cells, Adv Exp Med Biol 323, pp. 111-7.

Menzies, D.; Pai, M. and Comstock, G. (2007): Meta-analysis: new tests for the diagnosis of latent tuberculosis infection: areas of uncertainty and recommendations for research, Ann Intern Med 146 [5], pp. 340-54.

Meylan, E.; Tschopp, J. and Karin, M. (2006): Intracellular pattern recognition receptors in the host response, Nature 442 [7098], pp. 39-44.

Millington, K. A.; Innes, J. A.; Hackforth, S.; Hinks, T. S.; Deeks, J. J.; Dosanjh, D. P.; Guyot-Revol, V.; Gunatheesan, R.; Klenerman, P. and Lalvani, A. (2007): Dynamic

relationship between IFN-gamma and IL-2 profile of Mycobacterium tuberculosis-specific T cells and antigen load, J Immunol 178 [8], pp. 5217-26.

Mittrucker, H. W.; Steinhoff, U.; Kohler, A.; Krause, M.; Lazar, D.; Mex, P.; Miekley, D. and Kaufmann, S. H. (2007): Poor correlation between BCG vaccination-induced T cell responses and protection against tuberculosis, Proc Natl Acad Sci U S A 104 [30], pp. 12434-9.

Mollenkopf, H. J.; Grode, L.; Mattow, J.; Stein, M.; Mann, P.; Knapp, B.; Ulmer, J. and Kaufmann, S. H. (2004): Application of mycobacterial proteomics to vaccine design: improved protection by Mycobacterium bovis BCG prime-Rv3407 DNA boost vaccination against tuberculosis, Infect Immun 72 [11], pp. 6471-9.

Monack, D. M.; Mueller, A. and Falkow, S. (2004): Persistent bacterial infections: the interface of the pathogen and the host immune system, Nat Rev Microbiol 2 [9], pp. 747-65.

Moses, J (1930): Ter Totentanz von Lübeck, Co., Dr. Madaus &, Ed, Radebeul-Dresden.

Mosmann, T. R.; Cherwinski, H.; Bond, M. W.; Giedlin, M. A. and Coffman, R. L. (1986): Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins, J Immunol 136 [7], pp. 2348-57.

Mosmann, T. R. and Coffman, R. L. (1989): TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties, Annu Rev Immunol 7, pp. 145-73.

Mueller, H.; Detjen, A. K.; Schuck, S. D.; Gutschmidt, A.; Wahn, U.; Magdorf, K.; Kaufmann, S. H. and Jacobsen, M. (2008): Mycobacterium tuberculosis-specific CD4(+), IFNgamma(+), and TNFalpha(+) multifunctional memory T cells coexpress GM-CSF, Cytokine.

Nabel, G. J. (2002): HIV vaccine strategies, Vaccine 20 [15], pp. 1945-7.

North, R. J. (1998): Mice incapable of making IL-4 or IL-10 display normal resistance to infection with Mycobacterium tuberculosis, Clin Exp Immunol 113 [1], pp. 55-8.

Ogus, A. C.; Yoldas, B.; Ozdemir, T.; Uguz, A.; Olcen, S.; Keser, I.; Coskun, M.; Cilli, A. and Yegin, O. (2004): The Arg753GLn polymorphism of the human toll-like receptor 2 gene in tuberculosis disease, Eur Respir J 23 [2], pp. 219-23.

Orange, J. S. and Geha, R. S. (2003): Finding NEMO: genetic disorders of NF-[kappa]B activation, J Clin Invest 112 [7], pp. 983-5.

Orme, I. M. (2003): The mouse as a useful model of tuberculosis, Tuberculosis (Edinb) 83 [1-3], pp. 112-5.

Orme, I. M. (2006): Preclinical testing of new vaccines for tuberculosis: a comprehensive review, Vaccine 24 [1], pp. 2-19.

Ouellet, H.; Ouellet, Y.; Richard, C.; Labarre, M.; Wittenberg, B.; Wittenberg, J. and Guertin, M. (2002): Truncated hemoglobin HbN protects Mycobacterium bovis from nitric oxide, Proc Natl Acad Sci U S A 99 [9], pp. 5902-7.

Park, H. D.; Guinn, K. M.; Harrell, M. I.; Liao, R.; Voskuil, M. I.; Tompa, M.; Schoolnik, G. K. and Sherman, D. R. (2003): Rv3133c/dosR is a transcription factor that mediates the hypoxic response of Mycobacterium tuberculosis, Mol Microbiol 48 [3], pp. 833-43.

Park, L. S.; Morrissey, P. J.; Davison, B. and Grabstein, K. (1992): The role of IL-7 and its receptor in B-cell ontogeny, Adv Exp Med Biol 323, pp. 125-9.

Pym, A. S.; Brodin, P.; Majlessi, L.; Brosch, R.; Demangel, C.; Williams, A.; Griffiths, K. E.; Marchal, G.; Leclerc, C. and Cole, S. T. (2003): Recombinant BCG exporting ESAT-6 confers enhanced protection against tuberculosis, Nat Med 9 [5], pp. 533-9.

Rajalingam, R.; Mehra, N. K.; Jain, R. C.; Myneedu, V. P. and Pande, J. N. (1996): Polymerase chain reaction--based sequence-specific oligonucleotide hybridization analysis of HLA class II antigens in pulmonary tuberculosis: relevance to chemotherapy and disease severity, J Infect Dis 173 [3], pp. 669-76.

Rajalingam, R.; Singal, D. P. and Mehra, N. K. (1997): Transporter associated with antigen-processing (TAP) genes and susceptibility to tuberculoid leprosy and pulmonary tuberculosis, Tissue Antigens 49 [2], pp. 168-72.

Read, S.; Malmstrom, V. and Powrie, F. (2000): Cytotoxic T lymphocyte-associated antigen 4 plays an essential role in the function of CD25(+)CD4(+) regulatory cells that control intestinal inflammation, J Exp Med 192 [2], pp. 295-302.

Reece, S. T. and Kaufmann, S. H. (2008): Rational design of vaccines against tuberculosis directed by basic immunology, Int J Med Microbiol 298 [1-2], pp. 143-50.

Regner, M. (2001): Cross-reactivity in T-cell antigen recognition, Immunol Cell Biol 79 [2], pp. 91-100.

Reiling, N.; Holscher, C.; Fehrenbach, A.; Kroger, S.; Kirschning, C. J.; Goyert, S. and Ehlers, S. (2002): Cutting edge: Toll-like receptor (TLR)2- and TLR4-mediated pathogen recognition in resistance to airborne infection with Mycobacterium tuberculosis, J Immunol 169 [7], pp. 3480-4.

Robinson, H. L.; Hunt, L. A. and Webster, R. G. (1993): Protection against a lethal influenza virus challenge by immunization with a haemagglutinin-expressing plasmid DNA, Vaccine 11 [9], pp. 957-60.

Rock, K. L. and Shen, L. (2005): Cross-presentation: underlying mechanisms and role in immune surveillance, Immunol Rev 207, pp. 166-83.

Romagnani, S. (1991): Type 1 T helper and type 2 T helper cells: functions, regulation and role in protection and disease, Int J Clin Lab Res 21 [2], pp. 152-8.

Rook, G. A.; Steele, J.; Fraher, L.; Barker, S.; Karmali, R.; O'Riordan, J. and Stanford, J. (1986): Vitamin D3, gamma interferon, and control of proliferation of Mycobacterium tuberculosis by human monocytes, Immunology 57 [1], pp. 159-63.

Roupie, V.; Romano, M.; Zhang, L.; Korf, H.; Lin, M. Y.; Franken, K. L.; Ottenhoff, T. H.; Klein, M. R. and Huygen, K. (2007): Immunogenicity of eight dormancy regulon-encoded proteins of Mycobacterium tuberculosis in DNA-vaccinated and tuberculosis-infected mice, Infect Immun 75 [2], pp. 941-9.

Ruan, J.; St John, G.; Ehrt, S.; Riley, L. and Nathan, C. (1999): noxR3, a novel gene from Mycobacterium tuberculosis, protects Salmonella typhimurium from nitrosative and oxidative stress, Infect Immun 67 [7], pp. 3276-83.

Sambandamurthy, V. K. and Jacobs, W. R., Jr. (2005): Live attenuated mutants of Mycobacterium tuberculosis as candidate vaccines against tuberculosis, Microbes Infect 7 [5-6], pp. 955-61.

Saunders, B. M. and Britton, W. J. (2007): Life and death in the granuloma: immunopathology of tuberculosis, Immunol Cell Biol 85 [2], pp. 103-11.

Schaible, U. E. and Kaufmann, S. H. (2000): CD1 and CD1-restricted T cells in infections with intracellular bacteria, Trends Microbiol 8 [9], pp. 419-25.

Schaible, U. E.; Winau, F.; Sieling, P. A.; Fischer, K.; Collins, H. L.; Hagens, K.; Modlin, R. L.; Brinkmann, V. and Kaufmann, S. H. (2003): Apoptosis facilitates antigen presentation to T lymphocytes through MHC-I and CD1 in tuberculosis, Nat Med 9 [8], pp. 1039-46.

Schatz, A.; Bugie, E. and Waksman, S. A. (2005): Streptomycin, a substance exhibiting antibiotic activity against gram-positive and gram-negative bacteria. 1944, Clin Orthop Relat Res [437], pp. 3-6.

Schlesinger, L. S.; Bellinger-Kawahara, C. G.; Payne, N. R. and Horwitz, M. A. (1990): Phagocytosis of Mycobacterium tuberculosis is mediated by human monocyte complement receptors and complement component C3, J Immunol 144 [7], pp. 2771-80.

Schorey, J. S.; Carroll, M. C. and Brown, E. J. (1997): A macrophage invasion mechanism of pathogenic mycobacteria, Science 277 [5329], pp. 1091-3.

Seder, R. A.; Darrah, P. A. and Roederer, M. (2008): T-cell quality in memory and protection: implications for vaccine design, Nat Rev Immunol 8 [4], pp. 247-58.

Semmo, N.; Day, C. L.; Ward, S. M.; Lucas, M.; Harcourt, G.; Loughry, A. and Klenerman, P. (2005): Preferential loss of IL-2-secreting CD4+ T helper cells in chronic HCV infection, Hepatology 41 [5], pp. 1019-28.

Sherman, D. R.; Voskuil, M.; Schnappinger, D.; Liao, R.; Harrell, M. I. and Schoolnik, G. K. (2001): Regulation of the Mycobacterium tuberculosis hypoxic response gene encoding alpha -crystallin, Proc Natl Acad Sci U S A 98 [13], pp. 7534-9.

Shevach, E. M. (2000): Regulatory T cells in autoimmmunity*, Annu Rev Immunol 18, pp. 423-49.

Singh, C. R.; Moulton, R. A.; Armitige, L. Y.; Bidani, A.; Snuggs, M.; Dhandayuthapani, S.; Hunter, R. L. and Jagannath, C. (2006): Processing and presentation of a mycobacterial antigen 85B epitope by murine macrophages is dependent on the phagosomal acquisition of vacuolar proton ATPase and in situ activation of cathepsin D, J Immunol 177 [5], pp. 3250-9.

Singh, J. A.; Upshur, R. and Padayatchi, N. (2007): XDR-TB in South Africa: no time for denial or complacency, PLoS Med 4 [1], p. e50.

Skeiky, Y. A.; Alderson, M. R.; Ovendale, P. J.; Lobet, Y.; Dalemans, W.; Orme, I. M.; Reed, S. G. and Campos-Neto, A. (2005): Protection of mice and guinea pigs against tuberculosis induced by immunization with a single Mycobacterium tuberculosis recombinant antigen, MTB41, Vaccine 23 [30], pp. 3937-45.

Snyder, J. E.; Bowers, W. J.; Livingstone, A. M.; Lee, F. E.; Federoff, H. J. and Mosmann, T. R. (2003): Measuring the frequency of mouse and human cytotoxic T cells by the Lysispot assay: independent regulation of cytokine secretion and short-term killing, Nat Med 9 [2], pp. 231-5.

Sorensen, A. L.; Nagai, S.; Houen, G.; Andersen, P. and Andersen, A. B. (1995): Purification and characterization of a low-molecular-mass T-cell antigen secreted by Mycobacterium tuberculosis, Infect Immun 63 [5], pp. 1710-7.

Stahl, P. D. and Ezekowitz, R. A. (1998): The mannose receptor is a pattern recognition receptor involved in host defense, Curr Opin Immunol 10 [1], pp. 50-5.

Stalder, T.; Hahn, S. and Erb, P. (1994): Fas antigen is the major target molecule for CD4+ T cell-mediated cytotoxicity, J Immunol 152 [3], pp. 1127-33.

Stenger, S. (2001): Cytolytic T cells in the immune response to mycobacterium tuberculosis, Scand J Infect Dis 33 [7], pp. 483-7.

Sturgill-Koszycki, S.; Schlesinger, P. H.; Chakraborty, P.; Haddix, P. L.; Collins, H. L.; Fok, A. K.; Allen, R. D.; Gluck, S. L.; Heuser, J. and Russell, D. G. (1994): Lack of acidification in Mycobacterium phagosomes produced by exclusion of the vesicular proton-ATPase, Science 263 [5147], pp. 678-81.

Sun, Z. and Zhang, Y. (1999): Spent culture supernatant of Mycobacterium tuberculosis H37Ra improves viability of aged cultures of this strain and allows small inocula to initiate growth, J Bacteriol 181 [24], pp. 7626-8.

Szabo, S. J.; Sullivan, B. M.; Peng, S. L. and Glimcher, L. H. (2003): Molecular mechanisms regulating Th1 immune responses, Annu Rev Immunol 21, pp. 713-58.

Tang, D. C.; DeVit, M. and Johnston, S. A. (1992): Genetic immunization is a simple method for eliciting an immune response, Nature 356 [6365], pp. 152-4.

Torrelles, J. B.; Azad, A. K.; Henning, L. N.; Carlson, T. K. and Schlesinger, L. S. (2008): Role of C-type lectins in mycobacterial infections, Curr Drug Targets 9 [2], pp. 102-12.

Trunz, B. B.; Fine, P. and Dye, C. (2006): Effect of BCG vaccination on childhood tuberculous meningitis and miliary tuberculosis worldwide: a meta-analysis and assessment of cost-effectiveness, Lancet 367 [9517], pp. 1173-80.

Tsoukas, C. D.; Provvedini, D. M. and Manolagas, S. C. (1984): 1,25-dihydroxyvitamin D3: a novel immunoregulatory hormone, Science 224 [4656], pp. 1438-40.

Turner, J.; Frank, A. A.; Brooks, J. V.; Gonzalez-Juarrero, M. and Orme, I. M. (2001): The progression of chronic tuberculosis in the mouse does not require the participation of B lymphocytes or interleukin-4, Exp Gerontol 36 [3], pp. 537-45.

Ulrichs, T. and Kaufmann, S. H. (2006): New insights into the function of granulomas in human tuberculosis, J Pathol 208 [2], pp. 261-9.

Ulrichs, T.; Kosmiadi, G. A.; Trusov, V.; Jorg, S.; Pradl, L.; Titukhina, M.; Mishenko, V.; Gushina, N. and Kaufmann, S. H. (2004): Human tuberculous granulomas induce peripheral lymphoid follicle-like structures to orchestrate local host defence in the lung, J Pathol 204 [2], pp. 217-28.

van Pinxteren, L. A.; Cassidy, J. P.; Smedegaard, B. H.; Agger, E. M. and Andersen, P. (2000): Control of latent Mycobacterium tuberculosis infection is dependent on CD8 T cells, Eur J Immunol 30 [12], pp. 3689-98.

van Roon, J. A.; Glaudemans, K. A.; Bijlsma, J. W. and Lafeber, F. P. (2003): Interleukin 7 stimulates tumour necrosis factor alpha and Th1 cytokine production in joints of patients with rheumatoid arthritis, Ann Rheum Dis 62 [2], pp. 113-9.

Vandal, O. H.; Pierini, L. M.; Schnappinger, D.; Nathan, C. F. and Ehrt, S. (2008): A membrane protein preserves intrabacterial pH in intraphagosomal Mycobacterium tuberculosis, Nat Med.

Veenstra, H.; Crous, I.; Brahmbhatt, S.; Lukey, P.; Beyers, N.; van Helden, P. D. and Walzl, G. (2007): Changes in the kinetics of intracellular IFN-gamma production in TB patients during treatment, Clin Immunol 124 [3], pp. 336-44.

Veldhoen, M.; Hocking, R. J.; Atkins, C. J.; Locksley, R. M. and Stockinger, B. (2006): TGFbeta in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17-producing T cells, Immunity 24 [2], pp. 179-89.

Voskuil, M. I.; Schnappinger, D.; Visconti, K. C.; Harrell, M. I.; Dolganov, G. M.; Sherman, D. R. and Schoolnik, G. K. (2003): Inhibition of respiration by nitric oxide induces a Mycobacterium tuberculosis dormancy program, J Exp Med 198 [5], pp. 705-13.

Wayne, L. G. (1994): Dormancy of Mycobacterium tuberculosis and latency of disease, Eur J Clin Microbiol Infect Dis, 13, 11, 908-14.

Wayne, L. G. (1994): Dormancy of Mycobacterium tuberculosis and latency of disease, Eur J Clin Microbiol Infect Dis 13 [11], pp. 908-14.

Wayne, L. G. and Hayes, L. G. (1996): An in vitro model for sequential study of shiftdown of Mycobacterium tuberculosis through two stages of nonreplicating persistence, Infect Immun 64 [6], pp. 2062-9.

Weinrich Olsen, A.; van Pinxteren, L. A.; Meng Okkels, L.; Birk Rasmussen, P. and Andersen, P. (2001): Protection of mice with a tuberculosis subunit vaccine based on a fusion protein of antigen 85b and esat-6, Infect Immun 69 [5], pp. 2773-8.

Weir, R. E.; Brennan, P. J.; Butlin, C. R. and Dockrell, H. M. (1999): Use of a whole blood assay to evaluate in vitro T cell responses to new leprosy skin test antigens in leprosy patients and healthy subjects, Clin Exp Immunol 116 [2], pp. 263-9.

WHO (2008): Global Tuberculosis Control - surveillance, planning, financing, WHO Report 2008 [WHO/HTM/TB/2008.393].

Winau, F.; Kaufmann, S. H. and Schaible, U. E. (2004): Apoptosis paves the detour path for CD8 T cell activation against intracellular bacteria, Cell Microbiol 6 [7], pp. 599-607.

Winau, F.; Weber, S.; Sad, S.; de Diego, J.; Hoops, S. L.; Breiden, B.; Sandhoff, K.; Brinkmann, V.; Kaufmann, S. H. and Schaible, U. E. (2006): Apoptotic vesicles crossprime CD8 T cells and protect against tuberculosis, Immunity 24 [1], pp. 105-17.

Wong, K. C.; Leong, W. M.; Law, H. K.; Ip, K. F.; Lam, J. T.; Yuen, K. Y.; Ho, P. L.; Tse, W. S.; Weng, X. H.; Zhang, W. H.; Chen, S. and Yam, W. C. (2007): Molecular characterization of clinical isolates of Mycobacterium tuberculosis and their association with phenotypic virulence in human macrophages, Clin Vaccine Immunol 14 [10], pp. 1279-84.

Wucherpfennig, K. W. and Strominger, J. L. (1995): Molecular mimicry in T cell-mediated autoimmunity: viral peptides activate human T cell clones specific for myelin basic protein, Cell 80 [5], pp. 695-705.

Younes, S. A.; Yassine-Diab, B.; Dumont, A. R.; Boulassel, M. R.; Grossman, Z.; Routy, J. P. and Sekaly, R. P. (2003): HIV-1 viremia prevents the establishment of interleukin 2-producing HIV-specific memory CD4+ T cells endowed with proliferative capacity, J Exp Med 198 [12], pp. 1909-22.

Zhang, Y. (2005): The magic bullets and tuberculosis drug targets, Annu Rev Pharmacol Toxicol 45, pp. 529-64.

Zhang, Y. and Amzel, L. M. (2002): Tuberculosis drug targets, Curr Drug Targets 3 [2], pp. 131-54.

9 Appendix

9.1 Supplier List

Supplier	Location	URL
Adobe Systems GmbH	Ohmstrasse 1 D-85716 Unterschleißheim	www.adobe.de
Amersham Biosciences Europe GmbH	Munzinger Strasse 9, D-79111 Freiburg	www. amershambiosciences.com
Applied Biosystems	Division Headquarters, 850 Lincoln Centre Drive, Foster City, CA 94404, USA	www.appliedbiosystems.com
American Type Culture Collection (ATCC)	P.O.Box 1549, Manassas, VA 20108, USA	www.atcc.org
Bachofer GmbH now: Laborbedarf Saur	Wannweiler Straße 11, D- 72770 Reutlingen	www.h-saur.de
Bayer AG	D-51368 Leverkusen	www.bayer.com
B.Braun	Postfach 1120, D-34209 Melsungen	www.bbraun.de
Becton Dickinson GmbH (BD Biosciences): comprising products from Pharmingen and Clontech	Tullastrasse 8-12, D-69126 Heidelberg	www.bdbiosciences.com
Biochrom Labs, Inc.	1719 South 13th Street, P.O. Box 996, Terre Haute, IN 47808 USA	www.biochrom.com
Bio-Rad Laboratories GmbH	Heidemannstrasse 164 D-80939 München	www.bio-rad.com
Corning	Fisher Scientific GmbH, Im Heiligen Feld 17, D-58239 Schwerte	www.corning.com/lifesciences
ebioscience	AMDS und ERL, Postfach 1112, D-47552 Kranenburg	www.ebioscience.com
Eppendorf	Barkhausenweg 1, D-22339 Hamburg	www.eppendorf.com
Graph Pad Software, Inc.	11452 El Camino Real, #215 San Diego, CA 92130 USA	www.graphpad.com
Harvard Apparatus, Inc.	84 October Hill Road, Holliston, Ma. 01746, USA	www.harvardapparatus.com
Heraeus Instruments	Kendro Laboratory Products GmbH, Robert-Bosch-Strasse 1, D-63505 Langenselbold	www.heraeus-instruments.de
Hilgenberg GmbH	Strauchgraben 2, D-34323 Malsfeld	www.hilgenberg-gmbh.de
IKA Labortechnik GmbH & Co.KG	Janke & Kunkel-Strasse 10, D-79219 Staufen	www.ika.net
Invitrogen GmbH: comprising products from Invitrogen, NOVEX and Gibco	Technologiepark Karlsruhe, Emmy-Noether-Strasse 10, D-76131 Karlsruhe	www.invitrogen.com
Integra Bioscience GmbH	Ruhberg 4, D-35463 Fernwald	www.integra-bioscience.de
Ingenuity systems	Mountain View, California, USA	www.ingenuity.com
Jackson ImmunoResearch Europe Ltd	Unit 4, Northfield Business Park, Northfield Road, Soham, Cambridgeshire, UK CB7 5UE	www.jireurope.com
JPT Peptide Technologies	Volmerstrasse 5 (UTZ), D-12489 Berlin	www.jpt.com/index.htm
Lab-Therm Scientific		www.labtherm.co.uk
Leica Microsystems AG	Leica Mikrosysteme Vertrieb GmbH, Lilien-	www.leica-microsystems.com

Supplier	Location	URL
	thalstrasse 39-45, D-64625 Bensheim	
Memmert GmbH & Co. KG	Postfach 17 20, D-91107 Schwabach	www.memmert.com/de
Merck	Frankfurter Strasse 250, D-64293 Darmstadt	www.merck.de
Metabion GmbH	Lena-Christ-Strasse 44, D-82152 Martinsried	www.metabion.de
Microm International GmbH	Robert-Bosch-Strasse 49, D-69190 Walldorf	www.microm.de
Microsoft Deutschland GmbH	Katharina-Heinroth-Ufer 1, D-10787 Berlin	www.microsoft.de
Millipore GmbH	Am Kronberger Hang 5, D-65824 Schwalbach	www.millipore.de
Mobitec	Lotzestrasse 22°, D-37083 Göttingen	www.mobitec.de
Molecular Devices GmbH	Gutenbergstrasse 10, D-85737 Ismaning/ München	www.moleculardevices.com
Molecular Probes Europe BV	Poortgebouw, Rijnsburgerweg 10 2333 AA Leiden, The Netherlands	www.probes.com
New Brunswick Scientific	P.O. Box 4005, Edison, New Jersey, 08818-4005, USA	www.nbsc.com/
Nunc GmbH und Co.KG	Hagenauer Straße 21A, D-65203 Wiesbaden	www.nuncbrand.com
QIAGEN GmbH	QIAGEN Strasse 1, D-40724hilden	www.qiagen.com
R&D Systems GmbH	Borsigstrasse 7, D-65205 Wiesbaden	www.rndsystems.com
Roche Diagnostics GmbH Roche Applied Science	Sandhofer Strasse 116, D-68305 Mannheim	www.roche-applied-science.com
Carl Roth GmbH & Co.KG	Schoemperlenstrasse 1-5, D-76185 Karlsruhe	www.carl-roth.de
Sartorius AG	Weender Landstrasse 94-10, D-37075 Goettingen, Germany	www.sartorius.de
Savant	Savant, Farmingdale, NY, USA	www.schleicher-schuell.de
Schleicher & Schuell BioScience GmbH	Hahnestrasse 3, D-37586 Dassel/ Relliehausen	
Scientific Software Group	P.O. Box 708188, Sandy, Utah 84070, USA	www.scisoftware.com
Thermo Shandon, Inc.	171 Industry Dr., Pittsburgh, PA 15275, USA	www.shandon.com
Schütt Labortechnik	Rudolf-Wissell-Straße 11, D-37079 Göttingen	www.schuett-labortechnik.de/
Sigma-Aldrich Chemie GmbH	Eschenstrasse 5, D-82024 Taufkirchen bei München	www.sigmaaldrich.com
Zeiss: Unternehmensbereich Mikroskopie	Rankestr. 26, D-10789 Berlin	www.zeiss.de

9.2 HLA phenotyping

Donor	HLA-A	HLA-B	HLA-Cw	HLA-DRB1	HLA-DQA1	HLA-DQB1	HLA-DPB1
LTBI-A	0201 / 3201	4402 / 5101	0501 / 1502	0301 / 0401	0301 / 0501	02MS / 0302	0201 / 0401
LTBI-B	0201 / 6801	1302 / 5101	0602 / 1502	0404 / 0701	0201 / 0301	02MS / 0302	0401 / -
LTBI-C	0201 / 2501	1801 / 4402	0501 / 1203	0401 / 1101	0303 / 0505	0301 / -	0401 / -
LTBI-D	2902 / 6901	3508 / 5701	0701 / 1203	0403 / 0701	0201 / 0301	0302 / 0302	0401 / 0601
LTBI-E	0201 / 3201	0801 / 1501	0304 / 0701	0401 / 1302	0102 / 0301	0302 / 0604	0401 / 0402
LTBI-G	0101 / 6802	4402 / 5301	0401 / 0501	0402 / 1302	0102 / 0301	0302 / 0604	0401 / 0501
LTBI-H	0201 / 0301	3501 / 3901	0401 / 1203	0101 / 0404	0101 / 0301	0302 / 0501	0301 / 0402
LTBI-I	0201 / -	1501 / 1801	0304 / 1203	0401 / 0403	0301 / -	0302 / -	0401 / -
LTBI-J	0301 / 2402	1501 / 4001	0303 / 0304	1103 / 1501	0102 / 0505	0301 / 0602	0401 / 1001

MHC genotyping of LTBI recognizing Rv3407.

9.3 Zusammenfassung

Adaptive Immunantworten gegen *Mycobacterium tuberculosis (M. tuberculosis)* sind von entscheidender Bedeutung für die effektive Eindämmung des Erregers sowie den Schutz vor einer erneuten, sekundären Tuberkulose (TB). Obwohl Schlüsselfaktoren wie die T_h1 Zytokine IFNγ und TNFα bekannt sind, blieben Bemühungen zur Identifizierung eindeutiger immunologischer Parameter, welche ausschlaggebend für den Krankheitsverlauf sind, bislang erfolglos. Ein Großteil der infizierten Personen ist durch zelluläre Immunität in der Lage, die primäre TB zu bekämpfen und entwickelt darauf hin eine latente Tuberkulose Infektion (LTBI) während lediglich ~10% an aktiver TB erkranken. Ein besseres Verständnis der zugrunde liegenden Immunprozesse sowie die Identifikation projektiver Biomarker für TB sind zentrale Ziele dieser Arbeit.

Zur Bearbeitung dieser Fragestellungen wurden adaptive Immunantworten gegen *M. tuberculosis* in gesunden Probanden mit LTBI und Patienten mit aktiver TB analysiert. Hierfür wurde die Erkennung unterschiedlicher Proteine des Erregers durch die Messung IFNγ exprimierender $CD4^+$ $CD45RO^+$ Gedächtnis T Zellen untersucht. Eine Besonderheit war die Einbeziehung sogenannter Latenz-assoziierter Proteine, welche in Zusammenhang mit Dormanz und Reaktivierung des Bakteriums stehen. Optimierte Assay Bedingungen waren von Nöten, bevor adaptive Gedächtnis Antworten gegen diese Latenz-assoziierten Antigene mittels intrazellulärer Zytokinfärbung detektiert werden konnten. Dies Art von T Zell Antwort lies sich nicht im Rahmen konventioneller Kurz-Zeit Stimulationen nachweisen. 7 Tage *in vitro* Inkubation in Verbindung mit einer zweimaligen Restimulation belegten eine spezifische Erkennung durch $CD4^+$ $CD45RO^+$ T Zellen für die Mehrheit der getesteten Proteine bei Spendern mit LTBI. Der darauf folgende Vergleich zwischen Patienten mit aktiver TB und Personen mit LTBI zeigte signifikant höhere T Zell Antworten für 7 der 35 *M. tuberculosis* Proteine während LTBI.

Bemerkenswerterweise konnten spezifische T Zellen für eines der Protein, nämlich Rv3407, ausschließlich während LTBI gemessen werden und nicht bei Patienten mit aktiver TB. Diskriminanz Analysen zeigten, dass eine Unterscheidung zwischen LTBI und TB Patienten basierend auf T Zell Antwort gegen ausgewählte Latenz-assoziierte Antigene mit einer Genauigkeit von 82% möglich ist. Erneut erwies sich Rv3407 als der mit Abstand bedeutendste Faktor innerhalb der ausgewählten *M. tuberculosis* Proteine.

Durch den Einsatz synthetischer Peptidpools im Rahmen des neuen Assay konnten mehrere potentielle immunogene Epitope innerhalb von Rv3407 identifiziert werden. Darüber hinaus dokumentierte der Vergleich von T Zell Antworten nach Kurz-Zeit und Lang-Zeit Stimulation unterschiedliche Voraussetzungen für die *in vitro* Restimulation mit klassischen immundominanten Antigenen und Latenz-assoziierten Antigen. Diese Unterschiede führen zu der Hypothese, dass T Zellen mit Spezifität für Latenz-assoziierte *M. tuberculosis* Antigene einen anderen zellulären Phänotyp bzw. Reifungsgrad aufweisen.

Ein weiterer Schwerpunkt war die Studie zellulärer Zytokinmuster von *M. tuberculosis* spezifischen T Zellen zur Beurteilung der „Qualität" adaptiver Immunantworten und deren möglicher Beitrag zum Schutz vor dem Erreger. Diese Untersuchung belegte einen höheren Anteil Poly-Zytokin-produzierender T Zellen während LTBI im Vergleich zur aktiven TB. Insbesondere der Anteil von IFNγ/TNFα doppelt positiver (dp) sowie IFNγ/TNFα/IL2 trippel positiver (tp) T Zellen war hiervon betroffen. Die Gabe von rekombinantem IL7 in diesem Scenario führte zu einem weiteren absoluten und relativen Anstieg dieser T Zell Subpopulation. Die tatsächliche Relevanz dieser Beobachtungen *in vivo* sollte darauf hin im Rahmen neuer funktionaler *in vitro* Assays untersucht werden.
Die Infektion humaner Monozyten-abgeleiteter Makrophagen mit eGFP exprimierenden Mykobakterien bildete die Grundlage für die Erforschung der funktionalen Eigenschaften unterschiedlicher autologer T Zell Populationen. Vorversuche belegten das Potential der neuen Methode zur direkten Messung intrazellulärer Mykobakterien durch die Detektion $eGFP^+$ Makrophagen mittels Durchflusszytometrie. Darüber hinaus zeigte das neue *in vitro* Assay ein Dosis-abhängiges Abtöten von intrazellulären Bakterien, hervorgerufen durch autologe PBMC. Diese wurden zuvor entweder mit Hilfe von *M. tuberculosis* Lysat oder hohen Konzentrationen von IL2 aktiviert. Die Bestimmung der Anzahl lebensfähiger Makrophagen zeigte grundlegende mechanistische Unterschiede in dem erzielten Abtötungs-Effekt beider PBMC Typen. Der Kollateralschaden an Makrophagen war signifikant unterschiedlich und derartige Auswirkungen auf das Überleben der Wirts-Zellen hätten nicht durch eine konventionelle Bestimmung bakterieller Titer mittels CFU beobachtet werden können. Allerdings schmälerten beachtliche Assay-zu-Assay Variation den potentiellen Nutzen dieser vielversprechenden, neuen Methode. Vermutlich begründet sich diese Varianz in der Heterogenität der eingesetzten Makrophagen Population.

9.4 Publikationen

Schuck, S.D.*, Mahrenholz, C.C.*, Kretzschmar, I., Ay, B., Volkmer, R.; „An optimized SPOT synthesis protocol: High-throughput synthesis of peptides with authentic and free C-termini“ *Chem. Bio. Net.* (2005)

B. Ay, M. Streitz, P. Boisquerin, A. Schlosser, C.C. Mahrenholz, **S.D. Schuck**, F. Kern, R. Volkmer; „Sorting and Pooling strategy: a novel tool to map virus proteome for CD8 T-cell epitopes” *Biopolymers*, 88(1):64-75 (2007)

S.D. Schuck, H. Mueller, A. Gutschmidt, S. Leitner, S.H.E. Kaufmann, M. Jacobsen; „A novel rapid mycobacteria killing assay based on flow cytometry“ *Keystone Symp.*, 136-137 (2007)

H. Mueller, A.K. Detjen, **S.D. Schuck**, A. Gutschmidt, U. Wahn, K. Magdorf, S.H.E. Kaufmann; “Mycobacterium tuberculosis-specific $CD4^+$, $IFN\gamma^+$, and $TNF\alpha^+$ multifunctional memory T cells coexpress GM-CSF” *Cytokine*, 43(1): 143-148 (2008)

K. Magdorf, **S.D. Schuck**, S.Leitner, U. Wahn, S.H.E. Kaufmann, M. Jacobsen, „T cell responses against tuberculin and sensitin of children with tuberculosis and non-tuberculosis mycobacterial lymphadenopathy” *Clinical Microbiology and Infection*, accepted for publication (2008)

S.D. Schuck, H. Mueller, F. Kunitz, A. Neher, H. Hoffmann, K.L.C.M. Franken, D. Repsilber, T.H.M. Ottenhoff, S.H.E. Kaufmann, M. Jacobsen; “Identification of antigens specific for latent *Mycobacterium tuberculosis* infection by a novel T-cell assay” *The Journal of Immunology*, in review (2008)

9.5 Danksagung

Mein großer Dank gilt Herrn Prof. Dr. Stefan Kaufmann für die Möglichkeit, diese Arbeit in seiner Abteilung in einer großartigen wissenschaftlichen Umgebung anfertigen zu dürfen. Außerdem möchte ich ihm für das entgegengebrachte Vertrauen, seine wissenschaftlichen Denkanstößen sowie die Unterstützung, kompetente Betreuung und das Korrekturlesen danken.

Dr. Timo Ulrichs möchte ich für die sehr gute Einführung in die Arbeit am Max-Planck-Institut danken sowie Dr. Marc Jacobsen für die exzellente und äußerst produktive Betreuung. Ohne seinen fachlichen Rat und sein ausdauerndes Engagement wäre wohl so manches Projekt zum Scheitern verurteilt gewesen. In diesem Zusammenhang gilt mein besonderer Dank auch Sandra Leitner für ihre Unterstützung im Labor, die große Einsatzbereitschaft sowie ihren persönlichen Beitrag.

Auch meinen Mit-Doktoranden möchte ich an diese Stelle für ihre Freundschaft sowie den wissenschaftlichen und emotionalen Beistand danken. Explizit erwähnen möchte ich Henrik Müller und Juan Patron, die immer für einen Spaß zu haben waren und sich auch außerhalb des Instituts als gute Freunde bewiesen haben.

Selbstverständlich bin ich auch für die gute Zusammenarbeit mit der Lungenklinik Heckeshorn, dem Asklepios Klinikum München-Gauting sowie der Infektiologie der Charité dankbar. Ohne das große Engagement von Dr. Frank Kunitz und Dr. Albert Neher wäre die klinische Studie gewiss unmöglich gewesen.

9.6 Erklärung

Hiermit erkläre ich, die Dissertation selbständig und ohne unerlaubte Hilfe angefertigt zu haben.

Berlin, d. 02. September 2008